# DETERMINATION OF AMINO ACIDS IN FOODS AND BEVERAGES

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Tiivistelmä – Referat – Abstract

The literature study of this thesis focuses on the different analytical methods used to analyse amino acids in food and beverage samples. Amino acids are essential organic molecules and their concentrations in foods and beverages constitute, inter alia, the product's nutritional value, quality, freshness, and flavour. Amino acid analysis of foodstuff has various applications, which exploit several analytical methods. These reviewed methods are founded on academic articles published during the past two decades. This literature review discusses the different sample matrixes, sample preparation methods, ways to derivate analytes, and different separation and detection methods utilized in the recent amino acid studies.

The experimental part of this thesis was a modification of L-asparagine and L-aspartic acid test (L-Asp/L-AspAc) in Thermo Fisher Scientific Oy industrial R&D laboratory. An enzymatic photometric method is used to determine L-Asp/L-AspAc amino acids in food samples. The modification process entailed pre-testing of several candidate methods, from which the most suitable one was selected. The feasibility of the chosen test was affirmed before verification and validation of the modified test.



Avainsanat – Nyckelord – Keywords

Chromatographic analysis, amino acids, food samples, beverage samples, sample preparation, derivatisation, HPLC, GC, CE

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# Preface

The experimental part of this thesis was commissioned by the industrial research and development (R&D) laboratory of Thermo Fisher Scientific Oy in Vantaa during the summer of 2019.

I wish to thank the R&D team of Specialty Diagnostics Group for welcoming me so kindly and warmly on board. I have enjoyed working alongside you all and I am grateful for all the encouragement and advice given to me at the beginning of my budding career.

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# Abbreviations

| | |
|---|---|
| ACN | Acetonitrile |
| ALA | Alanine |
| AQC | 6-Aminoquinoyl-N-hydroxysuccinimidyl carbamate |
| ARG | Arginine |
| ASN | Asparagine |
| ASP | Aspartic acid |
| L-Asp/L-AspAc | L-asparagine/L-aspartic acid |
| BGE | Background electrolyte solution |
| BSTFA | N,O-Bis(trimethylsilyl)trifluoroacetamide |
| C1-3 | Candidate 1-3 |
| C8 | Octyl |
| C18 | Octadecyl |
| CE | Capillary electrophoresis |
| CI | Chemical ionisation |
| C&L | Classification and labelling |
| CMC | Critical micelle concentration |
| CMR | Carcinogenic, mutagenic, reprotoxic |
| CYS | Cysteine |
| CV | Coefficient of variation |
| DAD | Diode array detector |
| DC | Direct current |
| Dns-Cl | Dansyl chloride |
| DVB/CAR/PDMS | Divinylbenzene/Carboxen/Polydimethylsiloxane |
| ECF | Ethyl chloroformate |
| ECHA | European Chemical Agency |
| EI | Electron ionisation |
| EOF | Electro-osmotic flow |
| FID | Flame ionisation detector |
| FLD | Fluorescence detector |

| | |
|---|---|
| FMOC-Cl | 9-Fluorenylmethyl chloroformate |
| GC | Gas chromatography |
| GC-MS | Gas chromatography-mass spectrometry |
| GLN | Glutamine |
| GLU | Glutamic acid |
| GLY | Glycine |
| GOT | Glutamate-oxaloacetate transaminase |
| HCl | Hydrochloric acid |
| HILIC | Hydrophilic interaction liquid chromatography |
| HIS | Histidine |
| HPLC | High performance liquid chromatography |
| HPLC-MS | High performance liquid chromatography–mass spectrometry |
| ILE | Isoleucine |
| LC | Liquid chromatography |
| LED-IF | Light-emitting diode-induced fluorescence |
| LEU | Leucine |
| LIF | Laser-induced fluorescence |
| LLE | Liquid-liquid extraction |
| LOD | Limit of detection |
| LYS | Lysine |
| MCE | Microchip electrophoresis |
| MCF | Methyl chloroformate |
| MDH | Malate dehydrogenase |
| MEKC | Micellar electrokinetic chromatography |
| MeOH | Methanol |
| MET | Methionine |
| MS | Mass spectrometry |
| MTBSTFA | N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide |
| NaCl | Sodium chloride |
| NADH | Nicotinamide adenine dinucleotide |
| $NaHCO_3$ | Sodium bicarbonate |

| | |
|---|---|
| $NaH_2PO_4$ | Sodium dihydrogen phosphate |
| NaOH | Sodium hydroxide |
| NBD-Cl | 4-Chloro-7-nitrobenzofurazan |
| NBD-F | 4-Fluoro-7-nitrobenzofurazan |
| $NH_4OH$ | Ammonium hydroxide |
| OPA | O-Phthalaldehyde |
| PFBBr | Pentafluorobenzyl bromide |
| PHE | Phenylalanine |
| PITC | Phenylisothiocyanate |
| PRO | Proline |
| QC | Quality control |
| R1 | Reagent 1 |
| REACH | Registration, Evaluation, Authorization, and Restrictions of Chemicals |
| RF | Radio frequency |
| RP-LC | Reversed-phase liquid chromatography |
| RT | Rooms temperature |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SER | Serine |
| SIM | Selective ion monitoring |
| SLE | Solid-liquid extraction |
| SPME | Solid-phase microextraction |
| SVHC | Substances of very high concern |
| TFAA | Trifluoroacetic anhydride |
| THF | Tetrahydrofuran |
| THR | Threonine |
| TQMS | Tandem quadrupole mass spectrometer |
| TRP | Tryptophan |
| TYR | Tyrosine |
| UAE | Ultrasound-assisted extraction |
| UHPLC | Ultra-high-performance liquid chromatography |

| | |
|---|---|
| UV | Ultraviolet |
| VAL | Valine |

# 1. Introduction

The history of amino acids leads back to 1806 when two French chemists, Louis-Nicolas Vauquelin and Pierre Jean Robiquet, discovered and isolated the first amino acid, Asparagine, from the asparagus plant. Over the following decades, all the common amino acids that form proteins were discovered.[1] The first quantitative amino acid analysis was developed in 1948 by Stanford Moore and William H. Stein, who based their analysis method on postcolumn derivatisation of amino acids with ninhydrin.[2] In 1972, Moore and Stein alongside Christian Anfinsen were awarded the Nobel Prize in Chemistry for their work on ribonuclease molecules and connections between the amino acid sequence and the biologically active conformation. [3] Since then, the analysis of amino acids has developed fast due to the invention of new analysis methods.

The amino acids are important organic molecules, which form proteins and are involved in cellular growth and development.[4] Some of the amino acids are essential for humans, and amino acid deficiency may lead to serious health issues such as liver damage and cardiovascular diseases.[4] Furthermore, the determination of amino acid levels in foods and beverages is essential in order to assess the nutritional values and commercial quality of different food products.[4, 5] Correlation between amino acid levels and food spoilage has been studied, inter alia, by research group Bai et al.[6] who analysed amino acids from fish samples. Results verified that certain amino acids could be used as indicators to monitor food quality and safety. The amino acid analysis can be widely applied and it has been used, for example, to determine the botanical or geographical origin of food products and to detect adulteration.[7]

Amino acids can also be determined to evaluate the fermentation and taste of beverages.[8] Carvalho et al.[9] suggested that analysis of amino acid concentrations during beer's fermentation process may be more reliable than the monitoring of sugars, leading to better maintenance of beer quality. Research group Kelly et al.[10] studied the effects of added sulfur on amino acid levels in wines. The results proved that the inclusion of sulfur, which is commonly used as a preservative, lowers wine's amino acid concentration and thus reduces the aroma development during its maturing process.[10]

The amino acid analysis traditionally consists of hydrolysis, derivatisation, and chromatographic separation.[11] Sample preparation is an essential step for reliable analysis when studying challenging food matrixes. During the sample preparation the samples are homogenised, proteins are cleaved with hydrolysis to free bound amino acids, and analytes are derivatised. The derivatisation enables amino acid separation and detection with common analytical methods.[12] Different separation methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), ultra-high-performance liquid chromatography (UHPLC), and capillary electrophoresis (CE), are widely used. The most utilized detectors for amino acid analysis are ultra-violet, fluorescence, and spectrophotometric detectors.[11]

In the literature study of this thesis, different analysis methods used to analyse amino acids in food and beverage samples are reviewed. These methods include the different sample matrixes, sample preparation methods, ways to derivate analytes, and different separation and detection methods. All the discussed methods were published during the past two decades, namely between 2000 and 2020

## 2. Amino Acids

The amino acids are organic difunctional molecules with two important roles: they form proteins and are products of the cellular metabolism.[11] Thus amino acids can be found in every living cell in nature. All amino acids have a basic amino group, an acidic carboxyl group, and a unique side chain, which separates amino acids from each other.[13] The basic structure of an amino acid molecule is presented in Figure 1.

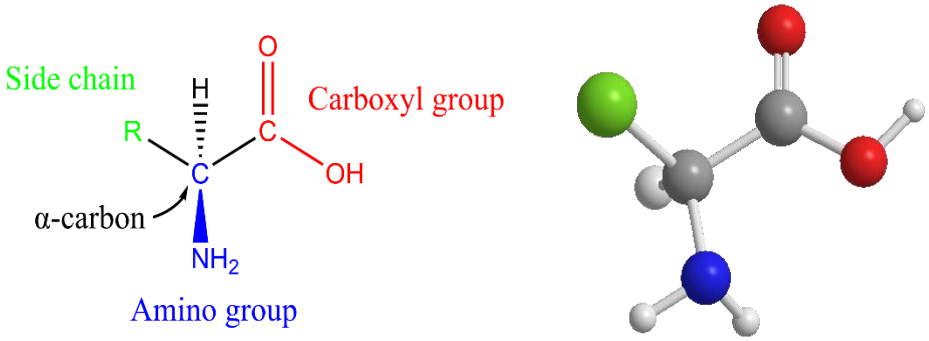


*Figure 1. Basic structure of amino acid molecule illustrated as skeletal formula (left) and 3D model (right).*

Because of the carbonyl and amino groups, all the amino acids are zwitterions when charged. In an acidic solution, amino acids are protonated and in a basic solution, the amino acids are deprotonated. When the solution's pH is between the pKa values of the amino acid, the molecule exists in its neutral dipolar zwitterion form.[13] The different forms of amino acid in different pH values are presented in Figure 2. The pKa1 and pKa2 values for each amino acid are listed in Table 1.

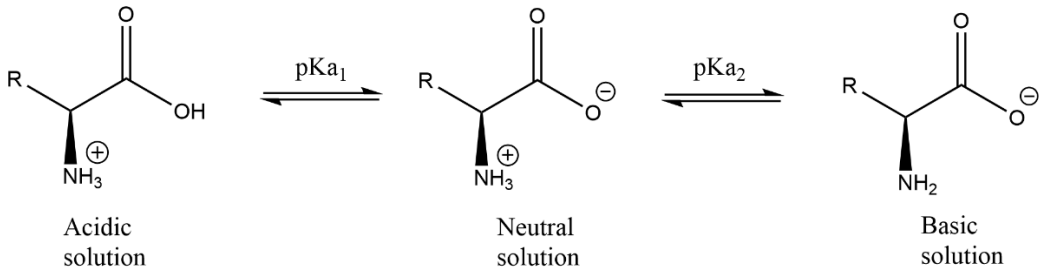


*Figure 2. Structure of amino acid depending on the pH of the solution*

The amino acids can form amide bonds between each other and make up long chains to form proteins and peptides. More than 700 amino acids have been found in nature.[13] However, proteins in humans are constructed with only 20 amino acids and these amino acids are alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.[11]

In Figure 3 are listed the 20 most common amino acids with their abbreviations and structures.[13]

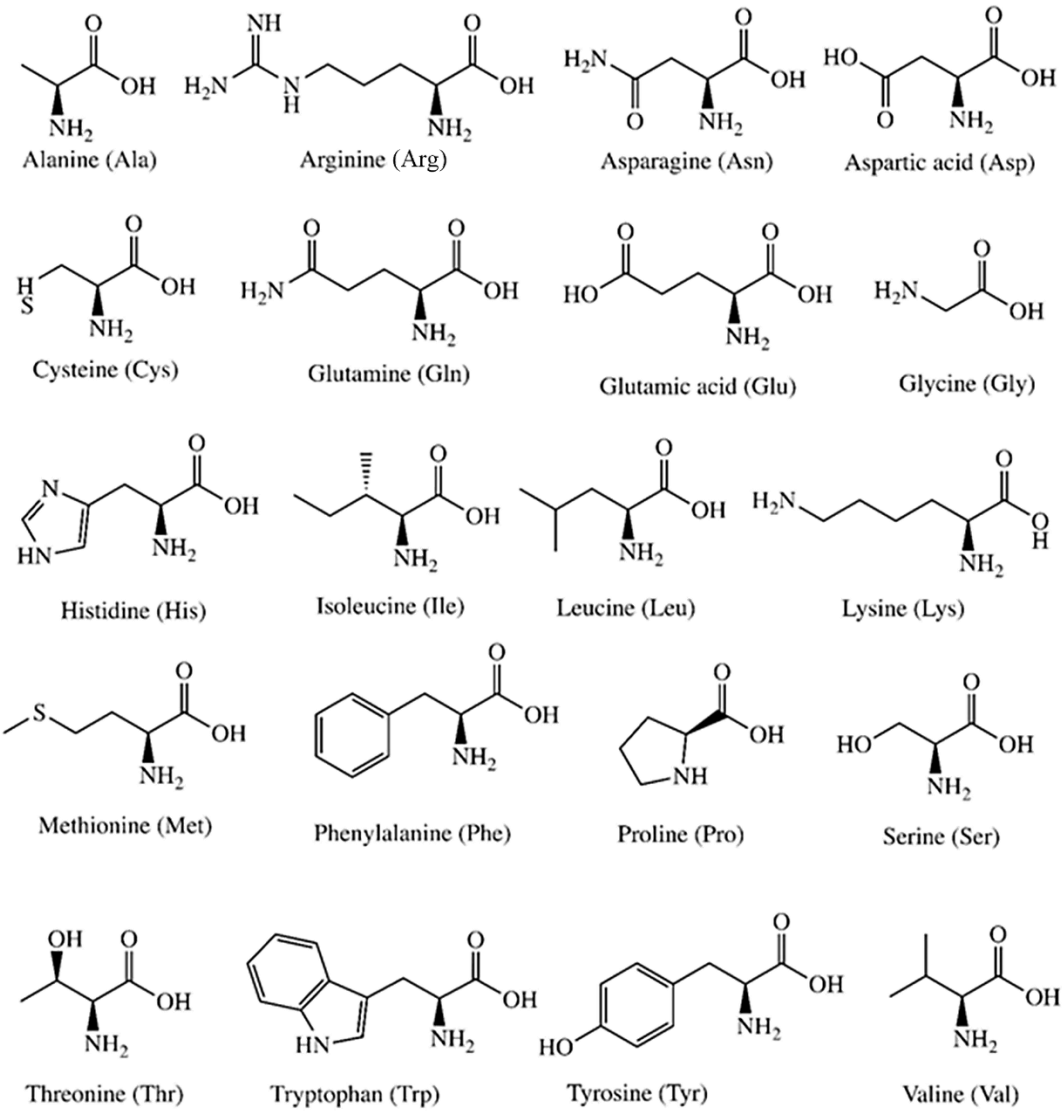


*Figure 3. Structures and abbreviations of the most common amino acids*

The structures in Figure 3 demonstrate that all the common amino acids are α-amino acids. They are also primary amino acids except for proline, which is a secondary amino acid. Furthermore, 19 out of the 20 common amino acids are chiral, the only exception being glycine. Two enantiomers of amino acids are possible, but nature uses only L-enantiomers to build up proteins.[11] D-amino acids do not offer the same nutritional value, and some can even be toxic to humans.[7]

The amino acids can be classified in many ways: by their polarity, charge, side-chain group, and their essentiality to humans.[11] The human body can synthesise only 11 out of the 20 needed amino acids; these are called non-essential amino acids. The rest of the amino acids must be obtained from food.[13] 20 of the most common amino acids, their pKa values, and different classifications are listed in Table 1.

*Table 1. Common amino acids, their pKa values and classifications based on analytes polarity, charge, structure of the side chain, and essentiality for humans.*

| **Amino Acid** | **$pKa_1$**[13] | **$pKa_2$**[13] | **Polarity** | **Charge** | **Structure of side chain** | **Essential** |
|---|---|---|---|---|---|---|
| Alanine | 2.34 | 9.69 | Non-polar | Neutral | Aliphatic | Non-essential |
| Arginine | 2.17 | 9.04 | Polar | Positive | Basic | Non-essential |
| Asparagine | 2.02 | 8.8 | Polar | Neutral | Amidic | Non-essential |
| Aspartic acid | 1.88 | 9.6 | Polar | Negative | Acidic | Non-essential |
| Cysteine | 1.96 | 10.28 | Non-polar | Neutral | Aliphatic | Non-essential |
| Glutamine | 2.17 | 9.13 | Polar | Neutral | Amidic | Non-essential |
| Glutamic acid | 2.19 | 9.67 | Polar | Negative | Acidic | Non-essential |
| Glycine | 2.34 | 9.6 | Non-polar | Neutral | Sulphur-containing | Non-essential |
| Histidine | 1.82 | 9.17 | Polar | Positive | Basic | Essential |
| Isoleucine | 2.36 | 9.6 | Non-polar | Neutral | Aliphatic | Essential |
| Leucine | 2.36 | 9.6 | Non-polar | Neutral | Aliphatic | Essential |
| Lysine | 2.18 | 8.95 | Polar | Positive | Basic | Essential |
| Methionine | 2.28 | 9.21 | Non-polar | Neutral | Sulphur-containing | Essential |
| Phenylalanine | 1.83 | 9.13 | Non-polar | Neutral | Aromatic | Essential |
| Proline | 1.99 | 10.6 | Non-polar | Neutral | Aliphatic | Non-essential |
| Serine | 2.21 | 9.15 | Polar | Neutral | Hydroxylic | Non-essential |
| Threonine | 2.09 | 9.1 | Polar | Neutral | Hydroxylic | Essential |
| Tryptophan | 2.83 | 9.39 | Non-polar | Neutral | Aromatic | Essential |
| Tyrosine | 2.2 | 9.1 | Polar | Neutral | Aromatic | Non-essential |
| Valine | 2.32 | 9.62 | Non-polar | Neutral | Aliphatic | Essential |

# 3. Sample preparation

Food is generally a complex matrix that may include many substances such as carbohydrates, proteins, fats, fibers, salts, vitamins, and minerals. If the food is processed, it may also contain preservatives, sweeteners, or other different additives. To analyse amino acids from different foods and beverages accurately, careful sample preparation is needed. During sample preparation, the analytes from solid food samples are transferred into liquid form, allowing analysis by chromatographic techniques.[14] Successful sample preparation concentrates the analytes of interest in the sample and removes interfering substances without contamination or analyte loss. This improves the separation and detection limit of the method.[14]

Amino acids can be present in food samples as free amino acids or bound to peptides and proteins. Free amino acids are present in different beverages and foods, where they affect the taste, smell, and nutritious value of the food.[15] For example, free L-glutamate increases the umami flavor, and free L-alanine provides sweetness to the flavour.[16] When the total amino acid concentration is determined, the amino acids bound in proteins and peptides are analysed alongside the free amino acids. To free the amino acids from proteins, the samples need to be hydrolyzed.[15] For total amino acid analysis, the hydrolysis can be done either during or after the sample preparation.

Common sample preparation steps for amino acid analysis of food include size reduction and homogenisation, extraction, concentration, and clean-up.[17] Depending on the sample matrix, the aforementioned steps mold the sample into an analysable form. For example, when preparing fresh fruit and vegetable samples, size reduction and homogenisation play an integral role in the sample preparation.[18] This step may include drying the sample for example in the oven[19] of freezing it[20]. After the sample is homogenised, the analytes are extracted from the sample matrix into the extraction solvent. Popular extraction methods for solid samples are solid-liquid extraction (SLE) and ultrasound-assisted extraction (UAE). The extraction step and sample hydrolysis may also be done simultaneously in a single step. These methods are discussed in more detail later in Chapter 3. After the extraction is completed, the sample is usually cleaned up and concentrated. The solid material is removed, for example, by centrifugation or filtration and the sample is dried to remove the excess solvent. Lastly, the dried sample is diluted into an appropriate solvent.

For liquid samples, the sample preparation is much simpler as the amino acids are already in the liquid phase. The samples are usually diluted and filtered prior to the analysis. Kelly et al.[10] analysed amino acids from wine and honey utilising simple sample preparation; wine and honey were diluted 1/10 (v/v) and (w/v) with distilled water, respectively, and filtered using a 0.45 µm membrane. Such sample preparation is straightforward, fast, and inexpensive as it does not require costly solvents. When analysing total amino acids, also the liquid samples need to be hydrolysed. If only free amino acid concentrations are studied, the sample's pre-treatment might be as simple as diluting the sample. This was the case in the Luo et al.[21] research, where the quality of the vinegar samples was studied.

## 3.1. Hydrolysis of Proteins

For analysing the total amino acid concentration in foods and beverages, the samples need to be hydrolysed. The samples' proteins are broken with hydrolysation reaction, where the addition of water breaks the amide bonds in proteins and yields free amino acids.[13] For foodstuff samples, classical acid hydrolysation is usually applied using 6M hydrochloric acid (HCl) at 110 °C for 24 h.[22] The procedure is widely used even though common amino acids vary in terms of stability when hydrolysed. Most amino acids are stable and have a good recovery, but for example, tryptophan is destroyed during acid hydrolysis.[15] During acidic hydrolysis, asparagine and glutamine are transformed into aspartic acid and glutamic acid respectively, and cysteine and methionine are partially oxidized.[15] For accurate determination of cysteine and methionine, the analytes need to be wholly oxidized into cysteic acid and methionine sulfone before hydrolysis.[23]

Albin et al.[22] studied the effects of hydrolysis time on the concentration of amino acids in different soybean products and reported that most of the amino acids reached their maximum concentration when hydrolysed for 24 h. However, serine's concentration was maximized when hydrolysis time was less than 24 h and valine and isoleucine reached their concentration maximum first after 24 h of hydrolysation.[22] The effect of hydrolysis time on mean yield for glycine, serine, and valine is demonstrated in Figure 4. Albin et al.[22] proposed the usage of correction factors when quantifying

amino acids with standard acidic hydrolysis procedures (24 h) in order to achieve more reliable and accurate results.

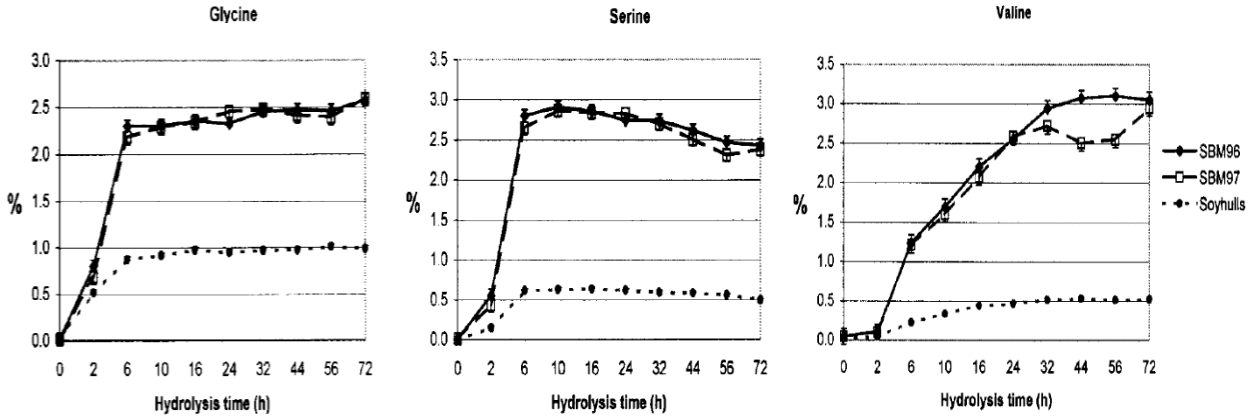


*Figure 4. Effect of hydrolysis time on the mean yield of Glycine, Serine and Valine from soybean meal from 1996 and 1997 and soybean hulls. Reprinted with permission form American Chemical Society.* [22]

Hydrolysis can also be done in alkaline conditions, but the method is mostly used for the determination of tryptophan, which is stable in basic conditions.[15] Hydrolysis can be performed for example with sodium hydroxide (NaOH) or lithium hydroxide.[15] Simionato et al.[24] compared three different hydrolysis procedures while determining amino acids from Brazil nut resin samples. The researchers discovered that while through basic hydrolysis only tryptophan was expected to be detected, also alanine, aspartic acid, histidine, lysine, phenylalanine, proline, threonine, tyrosine, and valine were detected with as good or better yield than with acid hydrolyzation. Also, leucine/isoleucine was detected, but the yield was low (47 %).[24] Interestingly, tryptophan was determined with the lowest yield of all analytes hydrolysed with the basic condition. The yield was only 33 % and the researchers considered that the used hydrolysis method (hydrolysis with barium hydroxide in 125 °C for 18 h) was suitable only for the qualitative determination of tryptophan.[24]

Simionato et al.[24] also used cation exchanger resin-mediated hydrolysis, where amide bonds were catalytically cleavaged by the H+ ions that were absorbed on a strong cation exchanger resin. This method is milder than standard acid hydrolysis and allows separation of hydrolysed analytes from other sample substances.[24] The method was briefly as follows: the cation exchanger resin was treated with 6M HCl solution and stirred for 1 h. The resin was washed and placed in a container

with grounded Brazil nut and 80:20 v/v water/ethanol solution. The sample was allowed to hydrolyse for 24 h at 110 °C. After hydrolysation, the sample was filtered, and the resin was washed with ammonium hydroxide solution to elute the amino acids. Finally, excess ammonia was evaporated, and the sample was diluted with deionised water.[24] Compared to the results gained with acid hydrolysis, the amino acid yields with resin hydrolysis were similar or better, and even alanine and serine could be detected. Still, the resin hydrolysis could not hydrolyse all the amino acids as the method was found to be inappropriate for asparagine, cysteine, glycine, glutamine, methionine, and tryptophan.[24]

In conclusion, there is no method to hydrolyse all the common amino acids simultaneously. The use of only one hydrolysing method restricts the total amino acid analysis in foods and beverages.

## 3.2. Solid-liquid extraction

Solid-liquid extraction is a straightforward extraction method used for solid samples. SLE is based on extracting analytes from solid samples into a chosen solvent. Extraction occurs when the analyte is more soluble in an extraction solvent than in the sample matrix, and therefore choosing the correct extraction solvent is essential for successful extraction.[17] This can be estimated by studying the analyte's partition coefficients (logP), which is defined with Equation 1.

*Equation 1. Partition coefficient*

$$logP_{OW} = \log \frac{[A]_O}{[A]_W} \quad (1)$$

where [A] is the analyte's concentration in n-octanol (O) and water (W).

When the analyte's partition coefficient value is positive, the analyte is more soluble in organic solutions and when the partition coefficient value is negative, the analyte is hydrophilic and prefers the water phase. According to PubChem's database[25] maintained by the National Center for Biotechnology Information, all the common amino acids have a negative partition coefficient and are thus more soluble in aqueous solutions. Suitable extraction solutions are, for example, water,

methanol, ethanol, and different mineral acids such as hydrochloride acid, perchloric acid, sulphuric acid, and trichloroacetic acid.[26]

The general SLE procedure is simple; the solid sample, that has been homogenised and as finely ground as possible to increase the sample's contact surface to the solvent, is immersed into the extraction solvent. Heating and mixing may be used to enhance extraction. A common SLE extraction apparatus is a reflux extractor, which was successfully utilised for tea sample extraction by Yan et al.[27] A schematic representation of the reflux extractor is presented in Figure 5. After the extraction process is completed, the solid sample is removed from the sample solution usually by filtration or centrifugation.

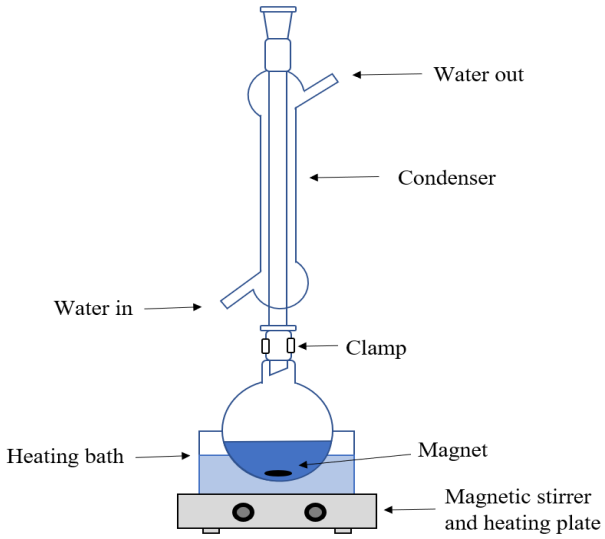


*Figure 5 Schematic representation of reflux extractor.*

Solid-liquid extraction, used in tea brewing and coffee making, is the most common extraction method in everyday life. It was also the most used extraction method among the presented studies, as most of the studies analysed solid food samples. Horanni et al.[28] extracted amino acids from ten different types of tea by stirring finely ground tea leaves with boiling water for 10 minutes. After the extraction, the tea leaves were removed with a filter.

Because hydrochloric acids can be used as an extraction solvent and are commonly used for acid hydrolyzation, it is possible to combine the extraction and hydrolysis steps. Several researcher groups took advantage of this opportunity, for example, Bosch et al.[23] analysed amino acids from

infant foods with the following sample preparation: commercial infant food was weighed into a Pyrex glass tube, where 6 M HCl was added and the sample was mixed. The tube was flushed with nitrogen for 1 minute to remove air. Next, the sample was hydrolysed at 110 °C for 23 h. After cooling to room temperature, the sample solution was filtered and diluted with deionised water.[23] The combination of extraction and hydrolysation saves labour, time, and reagents without affecting the accuracy of the method.[23]

### 3.3. Liquid-liquid extraction

In liquid-liquid extraction (LLE), the analytes are extracted from one liquid phase into another. Usually, extraction happens between an aqueous and organic solution. To extract analytes from an aqueous sample with an organic solvent, the analyte solubility in the organic solvent has to be better than in water.[17] Depending on the analyte's partition coefficients, the extraction solvent can be selected. As stated previously in Chapter 3.2, the free amino acids have negative partition coefficients and are more soluble in water than in organic solvent. Thus, the LLE was not used to extract amino acids from liquid sample matrixes, but the method was rather used in some cases after the amino acids had been derivatised. Derivatisation changes the analytes' properties and, for example, when analysing the amino acids with gas chromatography, the analytes are derivatized into less polar and more volatile compounds. After amino acid polarity is changed, it will be more soluble in an organic solvent and can be extracted with liquid-liquid extraction. Zhang et al.[29] analysed amino acids from selenium-enriched yeast and after the sample was derivatized with methyl chloroformate, the derivates were extracted with chloroform. After centrifuging the sample for 10 minutes, the chloroform layer was transferred into a sample vial for gas chromatography-mass spectrometry (GC-MS) analysis.

The liquid-liquid extraction is a simple extraction method, which does not require expensive laboratory equipment. The method is laborious if not automated; traditionally the extraction is done in an extraction funnel that is shaken to extract the analytes.[17] However, extensive usage of organic solvents and possible emulsion formation can be seen as the downsides of liquid-liquid extraction.[17] New extraction methods, such as solid-phase microextraction, have overcome these problems and offer a great extraction efficiency.[17]

## 3.4. Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) uses ultrasonic vibration to extract analytes from a solid sample into a liquid solution.[17] The method has an excellent extraction efficiency, which is based on the rarefaction and compression cycles of the ultrasound waves.[30] The rarefaction cycle produces cavitation bubbles, which are filled with solvent vapor. When the cavitation bubbles meet the compression cycle, they are compressed, which increases the bubbles' pressure and temperature substantially. The bursting bubbles cause shock waves that travel through the solvent and increase the solvent's penetration and mixing. The high effective temperature and the pressure increase solubility and diffusion, and enhance the method's extraction power.[30]

The UEA was used in a few of the represented studies. Corleto et al.[31] analysed free amino acids from fresh watermelon, cucumber, zucchini squash, yellow squash, calabaza squash, and celery. The sample preparation consisted of chopping the vegetables and passing them through a juicer. The vegetable juices containing parts of the pulp were sonicated for 30 minutes and centrifuged. The supernatants were filtered and diluted in deionised water. The matrix effect on the recovery of amino acids was studied and was reported to have minimal effect on the amino acid concentration. The recoveries for each sample matrix were found to be satisfactory.[31]

## 3.5. Solid-phase microextraction

Solid-phase microextraction (SPME) was used by Mudiam et al.[32] who combined the sample extraction, concentration, and injection into a single step. SPME is a fast and simple method that produces sensitive, selective, and repeatable results.[32] The method is solely based on sorption and desorption and it is well-suited for chromatographic analysis with the possibility for easy automation. The SPME procedure is based on inserting a sorbent-coated fiber into a sample or its headspace. The analytes are absorbed into the sorbent surface and can be injected into a GC analyser by placing the fiber into the injector port. The high temperature of the injector port releases the analytes from the fiber, which then can be used for the next sample. Mudiam et al.[32] used DVB/CAR/PDMS (65 µm) fiber for extracting the hydrolysed amino acids from soybean samples. The fiber was immersed in the sample for 30 minutes, after which the polar non-volatile

compounds were removed by rinsing the fiber with deionised water. After the fiber was dried, it was placed into a GC injection port for 5 minutes. Mudiam et al.[32] optimized SPME conditions and compared the effect of different fibers and results between headspace and immersion methods. The method mentioned above produced the best results with reported recoveries between 92–98 %.

# 4. Derivatisation of Amino acids

Derivatisation is needed when the analyte of interest does not have the desired properties for analysis. Such can be, for example, unsuitable chemical structure, volatility, solubility, melting point, or reactivity. Derivatisation can be utilized for two reasons, either to allow an analysis of compounds that are not initially suitable for analysis or to improve analysis, for example, by increasing chromatographic separation or detection.[12] Derivatisation reagents are used to form derivatives in order to modify analytes' properties.[33]

For amino acid analysis, sample derivatisation is almost always needed. When analysing with UV or fluorescence detector (FLD), derivatisation is necessary as most amino acids lack conjugated double bonds needed for detection.[20] Only Phenylalanine, Tryptophan, and Tyrosine have an aromatic group and can be detected with the aforementioned detectors without derivatisation. For better detectability, amino acids are converted into derivatives that have absorbance at the wanted wavelength or have fluorescence.[33] With derivatisation, the number of measurable analytes can be increased substantially.

Derivatisation is also used in the amino acid analysis to improve chromatographic separation. When analysing amino acids with gas chromatography, amino acids are usually derivatised into less polar and more volatile derivatives. This increases the interaction of the analyte with the column's active sites, increasing the method's sensitivity and decreasing possible tailing peaks.[14] When analysing amino acids with liquid chromatography, derivatisation enhances the separation by altering analytes polarity. Also, as discussed previously, amino acids are zwitterions and in ionic form in solutions. When separated in the reverse-phase liquid chromatography (RP-LC), ions

interact less with the stationary phase and elute quicker than in molecular form, possibly leading to incomplete separation and decreased sensitivity.[14] After amino acids are derivatised, they are no longer zwitterionic and the polarity has been altered to a more favourable one for RP-LC separation, which increases the selectivity and sensitivity of the method.[14]

Derivatisation can be performed either before, during, or after chromatographic separation. Terms precolumn-, oncolumn-, or postcolumn derivatisation are used to define at which point of the analysis the derivatisation occurs. The derivatisation process can also be done on-line or off-line and be automated. Precolumn derivatisation is traditionally used as it offers greater sensitivity than other methods.[15] In all amino acid studies where analytes were derivatised, precolumn derivatisation was utilized. Even though online and automated derivatisation offers better repeatability and has fewer problems with contamination than off-line derivatisation, it is not widely used due to the automation expenses. Of the represented studies, only three used automatized precolumn derivatisation. The most common derivatisation processes for HPLC, GC, and CE are discussed in more detail below. All the derivatisation processes used in the studied researches are presented in Table 2.

Where the analysis conditions allow, the amino acid analysis can be conducted even without derivatisation. For GC analysis, this is not possible as the analytes cannot be separated without derivatisation. However, when analysing amino acids with either HPLC or CE, derivatisation is not strictly needed, and the separation can be achieved by optimizing separation conditions. If analytes are not derivatised, the commonly used UV and fluorescence detectors cannot be utilized, as they do not detect the underivatised amino acids. In most studies where the analytes were not derivatised, mass spectrometers were used to detect the amino acids. Gökmen et al.[34] validated a rapid high performance liquid chromatography–mass spectrometry (HPLC-MS) method to analyse underivatised amino acids in 6 minutes. The mass spectrometry allowed sensitive detection of the amino acids without the need for lengthy sample preparation.[34]

*Table 2. General derivatisation processes for determination of amino acids in foods and beverages.*

| Analysis method | Derivatisation reagent | Abbr. | General Derivatisation process | Derivatisation conditions | Detector | Ref. |
|---|---|---|---|---|---|---|
| HPLC | o-Phthalaldehyde | OPA | OPA reagent was taken up by the autosampler and added to the sample vial. The mixture was incubated and analysed. | 1-2 min at 25°C | FLD | [10, 31, 35] |
| | Phenylisothio-cyanate | PITC | The sample was dried and reconstituted with PITC solution. Derivatisation was allowed to occur in dark. The sample was dried and reconstituted in ammonium acetate buffer. | 20-60 min at RT | UV | [19, 22, 33, 36] |
| | 9-fluorenylmethyl chloroformate | FMOC-Cl | The sample was mixed with potassium borate buffer and FMOC-Cl solution. Derivatisation was allowed to occur, and the reaction was stopped by the addition of acetic acid. The sample was filtered. | 10-20 min at RT | UV | [28, 37] |
| | 6-aminoquinoyl-N-hydroxy-succinimidyl carbamate | AQC | The sample was mixed with borate buffer and AQC solution and derivatisation was allowed to occur. The sample was heated to 55°C for 10 min. After cooling the sample was analysed. | 1 min at RT | FLD and UV | [23, 38] |
| | Dansyl chloride | Dns-Cl | The sample was mixed with NaOH solution, saturated $NaHCO_3$ solution, and Dns-Cl solution and derivatised in dark. Excess Dns-Cl was removed with $NH_4OH$. The sample was filtered. | 60 min at 40°C | UV | [6] |
| | Ethylaluminum-sesquichloride | EASC | The sample was mixed with borax buffer, ACN, and EASC solution and derivatised. The sample was filtered prior to analysis. | 10 min at 65°C | FLD | [4] |
| | 2-[2-(7H-dibenzo [a,g] carbazol-7-yl)- ethoxy] ethyl chloroformate | DBCEC-Cl | The sample was mixed with borate buffer, ACN, and DBCEC-Cl solution. The derivatisation was stopped by adding acetic acid. The sample was diluted with water and ACN before analysis. | 5 min at 30-40°C | FLD | [39] |
| GC | Ethylchloro-formate | ECF | The sample was derivatised with ECF. The derivatizing reagent was removed with nitrogen. Samples were dissolved in isooctane. | Seconds at RT | FID and MS | [5, 32, 40] |

*Table 2 continues.*

| Analysis method | Derivatisation reagent | Abbr. | General Derivatisation process | Derivatisation conditions | Detector | Ref. |
|---|---|---|---|---|---|---|
| GC | Methyl-chloroformate | MCF | The sample was mixed with methanol, pyridine, and MCF. Chloroform was added and the sample was mixed again. The sample was centrifuged, and the chloroform layer was transferred for analysis. | Seconds at RT | MS | [29] |
| | Trifluoroacetic anhydride | TFAA | Sample analytes were esterified by adding butanol and HCl for 1 h at 110°C. Trifluoroacetic anhydride was added and derivatisation was allowed to occur. The sample was dried and dissolved in ethyl acetate. | 20 min at 60°C | MS | [41] |
| | Pentafluorobenzyl bromide | PFBBr | The sample was mixed with phase-transfer catalysts (TBAB), phosphate buffer, and dichloromethane solution containing PFBBr. After derivatisation NaCl solution was added and dichloromethane layer was analysed. | 60 min at 100°C | FID and MS | [42] |
| CE | 4-Chloro-7-nitrobenzofurazan | NBD-Cl | The sample was mixed with borate buffer and NBD-Cl solution and derivatised. The reaction was stopped by adding water. The sample was diluted with water before analysis. | 30 min at 60°C | LIF and UV | [18, 27] |
| | 4-Fluoro-7-nitrobenzofurazan | NBD-F | The sample was mixed with NBD-F. The sample was diluted with borate buffer before analysis. | 3 min at 60°C | FLD | [43] |
| | Fluorescein isothiocyanate | FITC | The sample was mixed with borate buffer and FITC solution and derivatised. The reaction was stopped by adding water. The sample was diluted with water. | 4 h at 40°C | LIF | [44] |
| | Dansyl chloride | Dns-Cl | The sample was mixed with $NaHCO_3$ and Dns-Cl solution and derivatisation was allowed to occur in dark. The sample was dried 3 times with chloroform/methanol solution. Dried sample was reconstituted in borax and phosphate buffer solution. | 40 min at 65°C | UV | [20] |

ACN = Acetonitrile, FLD = Fluorescence detector, HCl = Hydrochloric acid, NaCl = Sodium chloride, $NaHCO_3$ = Sodium bicarbonate, $NH_4OH$ = Ammonium hydroxide, RT = rooms temperature, UV = Ultraviolet

## 4.1. Derivatisation in High Performance Liquid Chromatography

When analysing amino acids in foods by HPLC analysis, the most used derivatisation reagents are 6-aminoquinoyl-N-hydroxysuccinimidyl carbamate (AQC), Dansyl chloride (Dns-Cl), 9-fluorenylmethyl chloroformate (FMOC-Cl), Phenylisothiocyanate (PITC), and o-Phthalaldehyde (OPA).[15] The aforementioned derivatisation reagents were the most commonly used in the referred studies and their structures are presented in Figure 6.

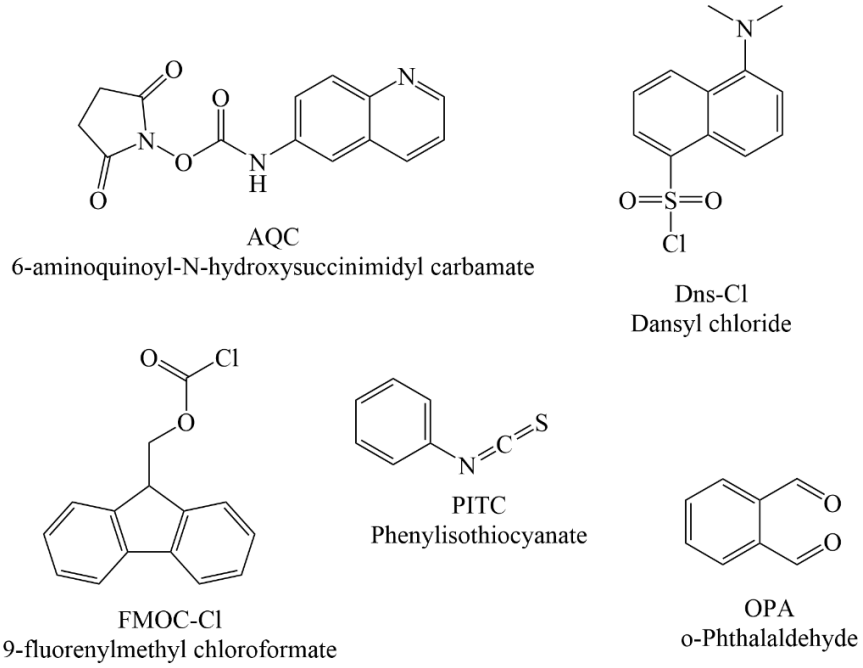


*Figure 6. Structures of the most common derivative reagents used in HPLC derivatisation*

From these common derivatisation reagents, PITC derivates are detectable with UV detector; the rest of the derivatisation reagents yield derivates that are also detectable with fluorescence detector.[23] Each of these derivatives has different advantages and disadvantages; for instance, OPA is a fast reagent, and derivatisation takes only minutes at room temperature, allowing derivatisation to be easily automated. The drawback is that OPA reacts only with primary amino acids and stopping the reaction must be carefully controlled for repeatable and reliable results. Three of the reviewed studies used OPA as a derivatisation reagent for automated precolumn derivatisation.[10, 31, 35] The derivatisation was reported to be fast; Corleto et al.[31] derivatised the vegetable sample in two minutes, after which the autosampler injected the sample for analysis.

Unlike OPA, PITC reacts with both primary and secondary amino acids, but with a longer derivatisation time of 20 to 60 minutes[19, 33]. An excess amount of PITC can interfere with analysis, so samples are often dried and reconstituted before analysis.[15] Because of the long derivatisation time and the sample clean-up step, PITC derivatisation is almost impossible to automatize. Researchers Albin et al.[22] analysed 15 amino acids from soybean products using PITC as a derivatisation reagent. The derivatisation was completed in 35 minutes at rooms temperature, after which the solvent was evaporated, and the sample was reconstituted in phosphate buffer and acetonitrile solution. The developed method was reported to be sensitive for all common amino acids.[22]

Similar to PITC, the Dns-Cl derivatises all the common amino acids, but the derivatisation time is even longer (60 min)[6] and is to be done in the dark due to the light-sensitivity of the Dns-Cl derivates. Another drawback is that Dns-Cl can react with both a hydroxyl and an amino group, decreasing the method sensitivity.[33] Also, excess reagents are to be removed before analysis for decreasing the interference. Compared to the aforementioned derivation regents, FMOC-Cl has a reasonable derivatisation time (10-20 min) at room temperature, and it derivatises both primary and secondary amino acids.[28, 37] However, FMOC-Cl derivatisation efficiency may decrease in presence of buffer salts, and excess derivatisation reagent is to be removed before analysis.[15, 23]

Of all the commonly used derivatisation reagents, AQC is the only one that overcomes all the previous problems. AQC reacts with all the common amino acids, and the derivates are stable at room temperature for one week.[23] The reaction time is fast, even less than one minute. Because the side products of the AQC derivatisation reaction have a different emission maximum than the derivatives, the analysis can be done without interference and a sample clean up. AQC derivatisation can be automated and the stability of derivates allow a sensitive offline derivatisation process. Two research groups, Bosch et al.[23] and Mayer et.al.[38] used Waters AccQ.Fluor™ reagent kit to derivatise infant food and cheese samples, respectively. Research groups reported that the developed methods were reproducible and accurate, and improved runtime and overall chromatographic performance.[23, 38] According to Mayer et.al "limits of detection and quantification obtained with AQC are better than those provided by other amino acid derivatizing agents." [38]

## 4.2. Derivatisation in gas chromatography

In GC analysis, common derivatisation methods used to transform analytes into more volatile and less polar molecules are either silylation or esterification/acylation.[45] Common silylation reagents for derivatisation are N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA). Both BSTFA and MTBSTFA are moisture sensitive and derivatisation is to be done under anhydrous conditions. Also, continuous heating is required.[45] Esterification and acylation are widely used for derivatisation of amino acids in aqueous samples. Of the studies using GC, all used esterification for derivatisation. The used derivatisation reagents were ethyl chloroformate (ECF), methyl chloroformate (MCF), trifluoroacetic anhydride (TFAA), and pentafluorobenzyl bromide (PFBBr). The structures are given in Figure 7

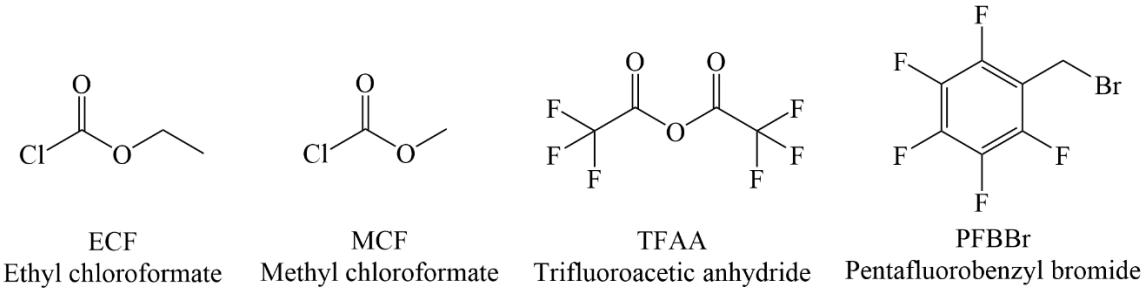


*Figure 7. Structures of the most common derivative reagents used in GC derivatisation*

The esterification is an easy and quick derivatisation method, where derivatisation takes only seconds.[15] After esterification, derivates are extracted or the sample is dried and reconstructed to avoid interference in detection. In the amino acid analysis of corn seeds, researchers Culeta et al.[41] used esterification and acetylation for the derivatisation of 17 amino acids. The pre-treated sample was first esterified with the addition of butanol and HCl. Then TFFA was added, and derivatisation was allowed to occur for 20 min at 60°C. Finally, the sample was dried and dissolved in ethyl acetate. The described derivatisation method is laborious, time-consuming, and includes many steps. The method also has drawbacks as it was reported to change asparagine and glutamine respectively into aspartic acid and glutamic acid.[41] For successful analysis, derivatisation should not lower the sensitivity or selectivity of the analysis.

Mudiam et al.[40] combined the derivatisation and extraction into a single step. Twenty amino acids were derivatised with ECF in the presence of pyridine and ethanol. Pyridine and ethanol were first added to the sample, after which ECF, acetonitrile, and trichloroethylene were added. The esterification reaction scheme is presented in Figure 8. After the amino acids were derivatised, they were extracted into an organic phase by sonication for 3 minutes. After centrifugation, the organic layer was analysed. The developed method was fast, easy, and had a low consumption of the extraction solvent. It had good repeatability and offered a great alternative to the traditional two-step derivatisation.[40]

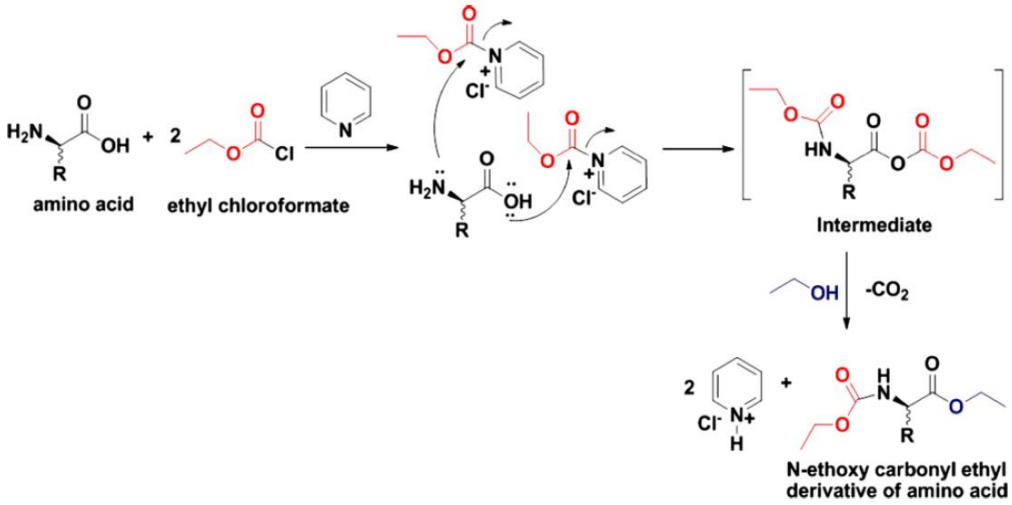


*Figure 8 Schematic representation of derivatisation of amino acid with ethyl chloroformate in the presence of pyridine and ethanol. Reprinted with permission form Elsevier.*[32]

When using phase-transfer catalyst the derivatisation and extraction steps can be combined. Scientists Fiamegos et al.[42] analysed amino acids from fruit juice and wheat flour and used PFBBr as a derivatisation reagent coupled with phase-transfer catalyst, tetrabutylammonium bromide, to transfer the derivates into the organic phase. Opposite to ECF, which derivatisation time is merely seconds at room temperature, derivatisation with PFBBr requires vigorous stirring for one hour at 100°C. After the derivatisation, the organic phase was separated and analysed.

## 4.3. Derivatisation in capillary electrophoresis

The need for amino acid derivatisation when analysing food samples with capillary electrophoresis is the same as in HPLC analysis; the amino acids are mostly invisible for UV and fluorescence detectors.[15] Because of this, the derivatisation reagents used in HPLC can also be employed for CE analysis. In CE, the oncolumn (oncapillary) derivatisation is more common than in HPLC analysis and can improve the separation in the capillary, which results in improved selectivity.[15] Depending on the CE method used for the analysis, the derivatisation procedures can vary even when using the same derivatisation reagent. For example, Omar et al.[18] and Yan et al.[27] used 4-Chloro-7-nitrobenzofurazan (NBD-Cl) as a derivatisation reagent but used capillary electrophoresis and micellar electrokinetic chromatography (MEKC) respectively as an electrophoretic technique. Both research groups optimized the derivatisation reaction conditions by studying the effect of pH, temperature, reaction time, and NBD-Cl concentration on derivatisation. Because of the two different electrophoretic techniques, the optimized derivatisation conditions differ from another. Omar et al.[18] performed the derivatisation in pH 8.5 with a reaction time of 30 min and temperature at 60°C with NBD-Cl concentration of 40 mM. Group Yan et al.[27] used instead the following conditions: pH of 8.5 with a reaction time of 60 min and temperature at 70°C with NBD-Cl concentration of 40 mM. Both of these methods were validated and had good linearity, precision, sensitivity, and accuracy.[18, 27]

Research group Ueno et al.[43] studied the amino acid amount in functional foods including sports beverages and tablet-form functional foods with microchip electrophoresis (MCE). They summarised that the derivatisation of amino acids with 4-Fluoro-7-nitrobenzofurazan (NBD-F) was fast as the reaction time was only 3 minutes at 60°C and the derivates had great stability when stored in the dark.[43] Despite the mentioned advantages, Ueno et al.[43] reported that the analysis method's sensitivity could be increased by optimising the separation parameters, as all the amino acids were not separated from one another. For example, amino acids Ile and Leu eluted at the same time.

Instead of derivatisation, Luo et al.[21] used copper (II) sulfate in the background electrolyte when analysing amino acids from vinegar with CE and UV detector. The copper (II) sulfate reacts with amino acids inside the capillary and forms coordination complexes [Cu(AA)n]2+, which provide strong UV absorption.[21] Compared to precolumn derivatisation, the developed method is faster, simpler, and uses fewer reagents. Luo et al.[21] reported that after diluting and filtering the vinegar sample, the analysis of 16 amino acids took a total of 40 minutes.

# 5. Analysis of Amino Acids

When determining several analytes simultaneously from a complex matrix, the analytes must be separated before detection for reliable results.[14] In analytical chemistry, chromatographic methods are widely used for quantitative and qualitative analysis. The most common analytical separation methods for determining amino acids are gas chromatography (GC) and high-performance liquid chromatography (HPLC), where the separation is based on the analyte's interactions with stationary and mobile phases.[14] Furthermore, methods using ultra-high-performance liquid chromatography (UHPLC) and capillary electrophoresis (CE) are also exploited. Amino acid analyses performed with the methods mentioned above are discussed in greater detail in the following chapters.

## 5.1. Gas Chromatography

Gas Chromatography is a common analysis method used to separate volatile and thermally stable analytes.[14] When the sample is injected into a heated injection port, the analytes evaporate and move through the capillary with the carrier gas. The carrier gas is an inert gas that acts as a mobile phase in the separation process. The most used carrier gases are nitrogen, hydrogen, and helium.[14] While the analytes are moving through the capillary column, they interact with the capillary's stationary phase. The stationary phase coats the inner surface of the column and usually consists of thermally stable polymers. Depending on the stationary phase's polarity, the analytes can be separated in their order of polarity.[14] After separation, the analytes are detected. Table 3 demonstrates the GC analysis conditions, including used columns, stationary phase, carrier gas, and detectors used to determine amino acids in food and beverage samples.

*Table 3. GC analysis conditions for determination of amino acids in foods and beverages.*

| Num. of AA | Matrix | Column | Stationary phase | Injection mode | Carrier gas | Flow rate (mL/ min) | Detector | LOD | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| 16 | Royal jelly | Fast analytical column (10 m ×0.25 mm) | NA | split | NA | 2 | FID | 0.009 mg/g | [5] |
| 17 | Corn seed | RTX-5 MS (30 m×0.25 mm, 0.25 µm | 95% dimethyl 5% diphenyl-polysiloxane | NA | Helium | 1 | MS | 0.001-0.01 mg/mL | [41] |
| 20 | Soybean | TG-17 MS (30 m×0.25 mm, 0.25 µm) | 50% phenyl 50% methyl-polysiloxane. | splitless | Helium | 1 | MS | 0.36–3.68 g/L | [40] |
| 16 | Soybean | TG-17 MS (30 m×0.25 mm, 0.25 µm) | 50% phenyl 50% methyl-polysiloxane | splitless | Helium | 1 | MS | 0.18-5.62 µg/L | [32] |
| 17 | Selenium enriched yeast | DB-5 MS (30 m×0.25 mm, 0.25 µm) | 95 % dimethyl 5% diphenyl-polysiloxane | splitless | Helium | 1.5 | MS | 0.01-0.20 nmol | [29] |
| 19 | Fruit juice and wheat flour | SPB-5 (30m×0.25mm, 0.25 µm) | 95% dimethyl 5% diphenyl-polysiloxane | splitless | Helium | 1 | MS<br>FID | 0.7-2.3 µmol/L<br>1.7-6.9 µmol/L | [42] |

Num. of AA = Number of analysed amino acids

FID = Flame ionisation detector, NA = not announced, MS = Mass spectrometer

As seen from Table 3, the most used column was 30 meters long, had an inner diameter of 0.25 mm, and was coated with a 0.25 µm thick polysiloxane layer. The dimensions of the column have a significant effect on the resolution; with a longer column, the analytes have more time to separate, and the resolution increases.[14] Similarly, if the film thickness is increased or the inner diameter is decreased, the analytes have more interaction with the stationary phase, and the resolution increases.[14] The longer the column is, the longer the analysis time is and higher pressure is required to achieve the wanted flow rate. Because of this, the resolution is seldom increased by lengthening the column.

Other factors affecting the separation efficiency are stationary phase composition, carrier gas and its flow rate, column temperature, and sample injection method. As summarised in Table 4, all the studies used polysiloxane-based stationary phases. The stationary phase's selectivity can be adjusted by replacing the methyl groups of the dimethylpolysiloxane with other functional groups, such as phenyl. Increasing the phenyl's amount in polysiloxane increases the stationary phase's polarity and the method becomes more selective for polar analytes.[14] All the studies used either the split or splitless injection methods. The splitless method enables quantitative analysis, whereas the split injection allows only qualitative analysis as some of the samples are lost during the injection.[14]

All the studies used helium as a carrier gas as it is more compatible than $H_2$ or $N_2$ with mass spectrometers. A flame ionisation detector (FID) and mass spectrometer (MS) were used to detect the amino acid concentration in the represented studies. Fiamegos et al.[42] analysed 19 amino acids from fruit juice and wheat flour samples using both GC-FID and GC-MS analysis with the same chromatographic conditions. Both methods were proven to be sensitive, simple, and accurate.[42] Of these two methods, the GC-MS was more sensitive than GC-FID, which is seen from the lower detection limit in Table 3.

The Flame ionisation detector ionises the separated organic analytes with a flame. The ionised analytes are directed in an electronic field towards collector electrodes, and as charged ions meet the electrodes, they generate an electrical current that can be measured.[14] FID has a very low background signal as pure hydrogen, carrier gas, and air/oxygen fuelled flame do not conduct electricity.[14] This leads to a good signal-to-noise ratio and very sensitive detection. In practice the FID can analyse almost any organic analyte eluting from the capillary.[14] For these reasons, the FID has become the most used detector coupled with GC.

In amino acid studies, mass spectrometry was the most used detection method. The mass spectrometry detects ionised analytes by their mass-to-charge ratio (m/z).[14] To get the analytes into a detectable form, they must be ionised. For GC-MS, mostly used ionisation methods are electron ionisation (EI) and chemical ionisation (CI) methods. Of these, chemical ionisation is a soft ionisation method, whereas the EI is a hard ionisation method that causes significant

fragmentation for the organic molecules. The EI was used as an ionisation method in all amino acid studies. The electrons were accelerated until the electrons had an energy of 70 eV. The electrons collide with the analytes and form analyte ions and fragments that can be detected by the mass spectrometer.[14]

All the GC-MS methods used a quadrupole mass analyser to analyse amino acids. The quadrupole analyser consists of four parallel metal rods in which the electric field is applied. The electric field is created by applying oscillating radio frequency (RF) voltage and a constant direct current (DC) voltage into the metal rods.[14] When ions enter the quadrupole, they start to oscillate between the metal rods. Only analytes with a wanted m/z ratio can pass through the quadrupole; ions with too light or heavy mass will eventually collide with one of the metal rods.[14] The quadrupole is highly selective, but this can be further enhanced by choosing selective ion monitoring (SIM) mode for analysis.[14] By selectively monitoring the wanted ions, the analysis is even more sensitive than FID. SIM mode was used by all the research groups to quantify the amino acids.

## 5.2. High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is based on the analyte's separation between a mobile and stationary phase. The term high-performance liquid chromatography (formerly known as high-pressure liquid chromatography) is used to distinguish the difference between traditional liquid chromatography that relies on gravity, and an instrumented liquid chromatography where high pressure is applied.[14] Compared to gas chromatography, the HPLC is more versatile as the only requirement for the analysis is to dissolve the analyte in a suitable solvent.[14] The HPLC is usually divided into categories depending on the separation mechanism. Reversed-phase liquid chromatography was used by researchers to analyse amino acids in food and beverage samples. In Table 4 are gathered the HPLC analysis conditions, including used columns, eluents, and detectors.

*Table 4. HPLC analysis conditions for determination of amino acids in foods and beverages.*

| Year | Number of analysed amino acids | Matrix | Column | Mobile phase A/B(/C) | Detector | LOD | Ref. |
|---|---|---|---|---|---|---|---|
| 2019 | 19 | Nitraria Tangutorum | Akasil C18 (200 mm × 4.6 mm, 5 µm) | $H_2O$:ACN (95:5) / ACN | FLD (λex = 262 nm, λem = 425 nm) | 0.13-1.13 nmol/L | [9] |
| 2019 | 21 | Commercial juices and fresh Watermelon, Cucumber, Celery, Zucchini Squash, Yellow Squash | Eclipse XDB C-8 (150 mm × 4.6 mm, 5 µm) | 20 mM sodium acetate buffer / ACN:MeOH:$H_2O$ (50:32:18) | FLD (λex = 340 nm, λem = 455 nm) | 0.02-0.19 ng/mL | [33] |
| 2019 | 19 | Monofloral bee pollen | Kinetex C18 (150 mm × 4.6 mm, 5 µm) | 60 mM ammonium formate / ACN:$H_2O$ (90:10) | FLD (λex = 265 nm, λem = 315 nm) | 0.05-0.55 µg/mL | [37] |
| 2019 | 17 | Antennaria Dioica | Zorbax AAA C18 (150 mm × 4.6 mm, 3 µm) | 40 mM $Na_2HPO_4$ / ACN:MeOH:$H_2O$(45:45:10) | FLD (λex = 265 nm) | NA | [46] |
| 2015 | 19 | Soybean | Luna C18 (150 mm × 2.1mm, 5 µm) | ACN / $H_2O$ (0.02% trifluoroacetic acid) | Orbitrap MS | 0.005-0.01 µg/mL | [47] |
| 2014 | 6 | Ginseng | Nova-Pak C18 (150 mm × 3.9 mm, 4 µm) | MeOH / 0.05M sodium acetate | DAD (λ=210-400 nm) | NA | [19] |
| 2013 | 19 | Tea | Kinetex C18 (100 mm × 2.6 mm,100 Å) | 0.1 M sodium acetate buffer (0.05% triethyl amine) / ACN:$H_2O$ (80:20) | UV (λ = 262 nm) | 0.057-0.534 mg/mL | [28] |
| 2011 | 20 | Potentilla Anserina | Hypersil BDS C18 (200 mm × 4.6 mm, 5 µm) | 30 mM ammonium + formic acid buffer:ACN (70:30) / ACN : 30 mM ammonium + formic acid buffer (50:50) / ACN | FLD (λex = 300 nm, λem = 395 nm) | 0.26-2.43 nmol/l | [39] |
| 2010 | 19 | Grape juice, wine, honey and physalis fruit | Equisil C18 (250 mm × 3 mm) | 50 mM sodium acetate buffer:MeOH (95:5) / MeOH:ACN (70:30) | FLD (λex = 330 nm, λem = 440 nm) | NA | [10] |
| 2006 | 17 | Infant foods | Nova-Pak C18 (150 mm × 3.9 mm, 4 µm) | AccQ.Tag™ Eluent A / ACN / $H_2O$ | FLD (λex = 250 nm, λem = 395 nm) | 0.016-0.367 µmol/L | [23] |

*Table 4 continues*

| Year | Number of analysed amino acids | Matrix | Column | Mobile phase A/B(/C) | Detector | LOD | Ref. |
|---|---|---|---|---|---|---|---|
| 2004 | 18 | Bovine and porcine gelatines | Knauer OPA special RP column (250 mm × 4 mm) | Sodium phosphate:MeOH (90:10) / MeOH:THF (97:3) | FLD (λex = 330 nm, λem = 450 nm) | NA | [35] |
| 2003 | 17 | Egg and soybean | Nova-Pak C18 (300 mm × 3.9 mm, 4 μm) | 20 mM $NaH_2PO_4$ (5% MeOH and 1.5% THF) / ACN:$H_2O$ (70:30) | UV (λ = 254 nm) | NA | [36] |
| 2001 | 17 | Breadfruit | Spherisorb C18 (250 mm × 4.6 mm, 5 μm) | 0.05M ammonium acetate / ACN:MeOH:$H_2O$ (44:10:46) | UV (λ = 254 nm) | NA | [33] |
| 2000 | 15 | Soybean products | Waters Pico-Tag C18 (300 mm × 3.9 mm, 4 μm) | 70 mM sodium acetate (2.5% ACN) / ACN:$H_2O$:MeOH (50:35:15) | UV (λ = 254 nm) | NA | [22] |

ACN = acetonitrile, C8 = octyl, C18 = octadecyl, DAD = diode array detector, FLD = Fluorescence detector, LOD = limit of detection, MeOH = methanol, NA = not announced, $NaH_2PO_4$ = sodium dihydrogen phosphate, MS = mass spectrometer, RP = reverse phase, THF = tetrahydrofuran, UV = ultraviolet, λex = excitation wavelength, λem = emission wavelength

In the RP-LC the stationary phase is less polar than the mobile phase. The analytes elute from the column in their order of polarity. As most polar analytes stay in the polar mobile phase and have little interaction with the stationary phase, they elute earlier than nonpolar analytes, which interact more with the stationary phase.

As seen from Table 4, the reverse phase separation was conducted either with octadecyl (C18) or octyl (C8) columns. The most used column dimensions were 150 mm × 4.6 mm with a particle size of 5 µm. By changing the column length and inner diameter, the separation efficiency and separation time can be modified. The backpressure limits the length and narrowness of the column. Also, the smaller the particle size is, the higher the backpressure gets.[14] When using smaller particles, the system needs to be able to withstand high pressures. With ultra-high-pressure liquid chromatography (UHPLC) the particle size can be reduced to under 2 µm. Studies that used UHPLC to analyse amino acids are introduced later in Chapter 5.3.

All the listed methods used gradient elution, where the mobile phase consists of two or more solvents. During the separation process, the mobile phase's composition is altered by changing the ratio of the elution solvents.[14] Usually, the separation is started with polar eluent A and after all the polar analytes have been eluted, the non-polar analytes are eluted with a non-polar solvent B. With gradient elution, the separation time is shorter than with isocratic separation, and the method allows better separation for multiple analyte mixtures.[14] In some cases, the gradient system can consist of three solvents, as seen in Table 4.

The most used detectors coupled with HPLC were ultraviolet detectors (UV) and fluorescence detectors (FLD). Only one research group used mass spectrometry (MS) for analysing the amino acids. The UV-detectors are specific, non-destructive detectors that are based on the analyte's ability to absorb electromagnetic radiation.[14] The amino acids that have been derivatised to absorb UV-light and separated with HPLC are detected by passing monochromatic light through the eluted mobile phase and measuring the absorbance. With Beer-Lambert law (Equation 2), the concentration is calculated from the absorbance.[14]

*Equation 2. Beer-Lambert law*

$$A = \varepsilon bc \quad (2)$$

where A is absorbance, ε is molar absorptivity, b is path length, and c is sample concentration.

The diode array detector (DAD) is one type of UV-detector. Instead of measuring only one wavelength at a time, the DAD measures multiple wavelengths simultaneously. A diode array detector was successfully used for amino acid analysis by Han et al.[19] with a wavelength range of 210-400 nm. Compared to the traditional monochromatic UV-detector, the DAD represents the results in three dimensions. Absorbance is expressed both as a function of wavelength and time. This gives an advantage over a traditional UV-detector. Overall, the UV-detector is a good and reliable detector.

The other popular detector among the amino acid analysis was the fluorescence detector. FLD is a specific and non-destructive detector, which excites the analytes with electromagnetic radiation.[14] The excited molecule can lose the extra energy in multiple ways, such as through colliding with other molecules or by phosphorescence. If the excited analyte loses some of its energy and moves to its lowest vibrational level of the excited state, (S1) and furthermore relaxes to the ground state (S0) by transitioning between two states that have the same spin (i.e. singlet to single), a photon with a longer wavelength than the absorbed photon will emit.[14] The process is presented schematically in Figure 9.

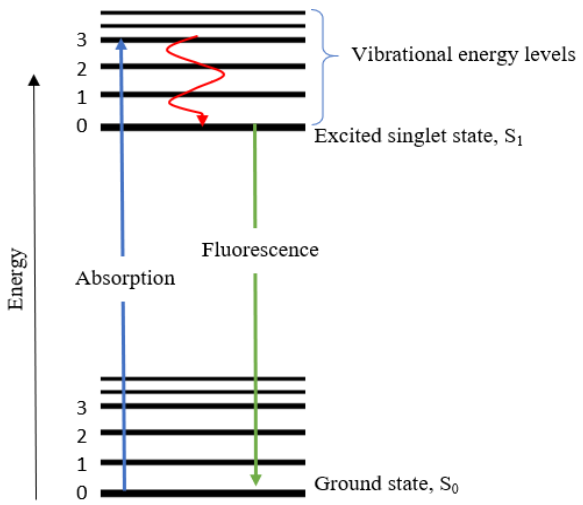


*Figure 9. Schematic representation of molecules excitation and fluorescence.*

Compared to the UV-detector, the FLD apparatus consists of two optical systems: one that emits monochromatic light at the wanted wavelength into the sample, and the other that filters the emitting light from the sample at the wanted wavelength and directs it into a photomultiplier tube. The FLD has several orders of magnitude greater sensitivity than the UV-detector.[14] The downside is that few compounds are naturally fluorescent, and most analytes need to be derivatised before detection.

## 5.3. Ultra-High-Performance Liquid Chromatography

Ultra-high-performance liquid chromatography (UHPLC) is an improved analysis method with enhanced separation efficiency, resolution, and analysis time compared to the HPLC analysis.[14] UHPLC is based on the same separation principle as the HPLC. However, the difference between these methods is that in comparison to the HPLC, the UHPLC uses smaller particles inside the column, which leads to very high pressure.[14] The pressure gets so high that the traditional HPLC instrument could not operate under it. The maximum backpressure in HPLC is around 400 bars, whereas the UHPLC is made to withstand a 1000 bar backpressure.[14, 38] The UHPLC method typically uses particles with a diameter smaller than 2µm. The smaller particle size enhances the

separation efficiency, which increases resolution, selectivity, and sensitivity. In Table 5 are gathered the UHPLC analysis conditions for analysing amino acids in food and beverage samples.

*Table 5. UHPLC analysis conditions for determination of amino acids in foods and beverages.*

| Year | Num. of AA | Matrix | Column | Mobile phase A/B | Detector | LOD | Ref. |
|---|---|---|---|---|---|---|---|
| 2019 | 10 | Tuna and Mahi-mahi | Kinetex C18 (50 mm × 2.1 mm, 1.3 μm) | $H_2O$ (0.1% FA) / ACN (0.1% FA) | UV (λ = 254 nm) | 0.01-0.28 ppm | [6] |
| 2013 | 23 | Ziziphus Jujuba | ACQUITY HILIC Amide column (100 mm × 2.1 mm, 1.7 μm) | 10 mM ammonium formate (0.15% FA) / ACN (2mM ammonium formate, 0.15% FA) | MS | 0.17-77.75 ng/ml | [48] |
| 2013 | 18 | Cheese | Acquity UPLC C18 (50 mm × 2.1 mm, 1.7 μm) | AccQ.Tag™ Eluent A / ACN:$H_2O$ (60:40) | UV (λ = 254 nm) | 0.8-3.7 mg/100g | [38] |
| 2012 | 22 | Apple juice, Honey, Wine | Atlantis HILIC silica column (150 mm × 2.1 mm, 3 μm) | ACN / $H_2O$ (0.1% FA) | MS | 0.1-46.7 ng/ml | [34] |

Num. of AA = Number of analysed amino acids
ACN = acetonitrile, C18 = octadecyl, FA = formic acid, HILIC = hydrophilic interaction liquid chromatography, LOD = limit of detection, MeOH = methanol, MS = mass spectrometer, TQMS = tandem quadrupole mass spectrometer, UV = ultraviolet

Table 5 shows that the column dimensions are the most significant difference between UPHLC and HPLC analysis methods. In addition to the smaller particle size, also shorter columns are used. Because the separation is so efficient, the column can be shorter, which leads to shorter analysis times. The analysis can be done even in ten minutes, compared to the HPLC, which usually has an analysis time between 20 and 60 minutes.[4, 39]

Guo et al.[48] analysed simultaneously 23 free underivatised amino acids from Ziziphus Jujuba fruit with the UPHLC-MS method in 12 minutes. The method separated the analytes with a HILIC Amide column with a particle size of 1.7 µm. Hydrophilic interaction liquid chromatography (HILIC) is a sub-method to normal-phase HPLC, where the separation is based on hydrophilic interactions between the polar stationary phase and polar analytes.[48] The HILIC-UHPLC-MS method was found to be sensitive, reliable, and simple with amino acids recoveries between 93.5% and 103.6% [48]

## 5.4. Capillary Electrophoresis

Capillary electrophoresis is a separation method based on the ionic analytes' movement in an electric field. The CE resembles the GC and HPLC methods as the separation occurs inside a capillary column through which the mobile phase flows.[14] However, the separation method differs from chromatographic separation as the separation is not based on the analyte's interactions between mobile and stationary phases.[14] The basic analysis principle is as follows: The analytes are introduced into an uncoated capillary column and the ends of the capillary column are placed into buffer solution reservoirs. Into the reservoirs are placed anode and cathode, and high voltage is applied between electrodes.[14] The created electric field separates the analytes inside the capillary as the analytes migrate towards the cathode, due to their different electrophoretic mobilities.[14]

The buffer solution inside the capillary is called background electrolyte solution (BGE). The electrolytes in the BGE solution form a diffuse double layer on top of the free silica surface.[14] The double layer consists of cations from BGE solution, that cover the negatively charged silica surface. When the voltage is applied, the cations in the double layer start to move towards the cathode and move the BGE solution toward the cathode as they move. The diffuse double layer movement that also causes the BGE solution's movement is called electro-osmotic flow (EOF).[14] The EOF flow has a blunt flow profile compared to the chromatographic parabolic flow profile. Due to the bluntness, the CE methods have very narrow analyte peaks and good separation compared to the HPLC method.[14] Analytes with both positive and negative charges can be analysed simultaneously with CE as the EOF moves all the analyte ions towards the detector. Cations move naturally towards the cathode and arrive first to the detector. Anions move toward

the anode but arrive eventually at the detector as the EOF has greater velocity than the analytes and moves even anions towards the cathode.[14] Capillary electrophoresis is an efficient, fast, and environmentally friendly analysis method due to its low reagent consumption.[20] CE has been broadly used for amino acid analysis in food and beverage samples.

Table 6 lists different CE methods used for amino acid analysis. Besides the traditional capillary electrophoresis method, micellar electrokinetic chromatography (MEKC) and microchip electrophoresis (MCE) were used as analysis methods. MEKC is based on the same instrumentation as CE, but the separation principle is different. In MEKC, surfactants are added to the buffer solution where they form micelles. As the micelles travel through the capillary, they act as a pseudo stationary phase and enable neutral analytes' separation alongside ionic analytes.[14] The resolution can be significantly affected by optimizing the separation conditions and choosing a suitable surfactant. Yan et al.[27] investigated common surfactants sodium dodecyl sulphate (SDS) and Brij35 to obtain optimum analysis conditions. They discovered that the use of Brij35 resulted in a better separation in a shorter time than using SDS.

Separation can also be affected by optimizing the column dimensions. Table 6 lists the used column in amino acid analyses. The Table specifies the column's total length, length to the detector, and capillary's inner diameter. The resolution and separation efficiency can be improved by increasing the capillary's length or decreasing the inner diameter.[14] Luo et al.[21] studied the effects of capillary's inner diameter (id.) on the separation and discovered that while using a column with 75 µm id. the amino acids were not separated from each other, as some of the peaks were overlapping.[21] By reducing the inner diameter to 50 µm, a good separation was achieved.

*Table 6. CE analysis conditions for determination of amino acids in foods and beverages.*

| Year | Num. of AA | Matrix | Column | Background electrolyte solution | Separation voltage | Method | Detector | LOD | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| 2018 | 16 | Vinegar | fused silica (73 / 65 cm x 50 µm) | 50 mM copper (II) sulfate solution | 22.5 kV | CE | UV (354 nm) | 0.13 - 0.25 g/mL | [21] |
| 2018 | 6 | Brewery wort | fused silica (60 / 51.5 cm x 50 µm) | 50 mM phosphate buffer (0.4 mM cetyltrimethyl-ammonium bromide) | 20 kV | CE | DAD (200-230 nm) | 0.75 - 15.0 µg/ml | [9] |
| 2017 | 6 | Potato, eggplant, chickpeas, wheat flour, durra flour | fused silica (40 / 30 cm x 50 µm) | 100 mM borate buffer | 25 kV | CE | UV (475 nm) | 0.32 - 0.56 mg/L | [18] |
| 2015 | 16 | Turtle jelly, fish, egg, pork, chicken | fused silica (60.2 / 50 cm x 50 µm) | 20 mM borate and 20 mM phosphate buffer (0.1M SDS, 6% MeOH) | 25 kV | MEKC | UV (214 nm) | 1.69 - 9.15 µg/mL | [20] |
| 2014 | 15 | Tea leaves | fused silica (50.2 / 40.0 cm x 75 µm) | 20 mM sodium borate (20 mM Brij 35, 10% ACN) | 20 kV | MEKC | LIF (λex = 488 nm, λem = 520 nm) | 0.1 - 100 ng/mL | [27] |
| 2013 | 11 | Pomegranate juice | fused silica (60 / 45 cm x 50 µm) | 50 mM borate (5 mM SDBS, 10 mM b-cyclodextrin) | 25 kV | MEKC | LIF (λex = 488 nm) | 0.84 - 3.99 nM | [44] |
| 2008 | 11 | Brazil nut | fused silica (80 / 70 cm x 50 µm) | H2O:MeOH (80:20) (0.8% formic acid) | 30 kV | CE | MS | 16 - 172 µmol/L | [24] |
| 2008 | 18 | Sports beverages, jelly-form sports beverages, tablet-form functional foods | - | 100 mM borate buffer, 30 mM SDS | - | MCE | LED-IF (470 nm) | NA | [43] |

Num. of AA = number of analysed amino acids, ACN = acetonitrile, CE = capillary electrophoresis, DAD = diode array detector, LED-IF = light emitting diode-induced fluorescence, LIF = laser induced fluorescence, LOD = limit of detection, MCE = microchip electrophoresis, MEKC = micellar electrokinetic chromatography, MeOH = methanol, MS = mass spectrometer, SDBS = sodium dodecylbenzene sulphonate, SDS = sodium dodecyl sulphate, UV = ultraviolet, λex = excitation wavelength, λem = emission wavelengt

The capillary electrophoresis is usually combined with a UV or fluorescence detector, and the detection can be performed through the capillary by removing a short section of the protective coating of the capillary.[14] Because of the narrowness of the capillary, the light beam's path length is short, which decreases sensitivity.[14] From Table 6 it can be summarised that UV detector was the most used detection method, when analysing amino acids. Other detection methods were laser-induced fluorescence detection (LIF), light-emitting diode-induced fluorescence detection (LED-IF), and spectrometry. In the LIF and LED-IF detection, the fluorescence is either induced by laser or led light. Researchers Ueno et al.[43] used microchip electrophoresis (MCE) with an LED-IF detector to analyse 18 amino acids from different functional foods. The MCE is a miniature CE separation method that was found to have several advantages. The method was reported to be simple, inexpensive, and extremely fast, with total analysis time, including the sample preparation, of less than 10 minutes.[43]

# 6. Summary of literature study

In the literature part of this thesis, amino acid analysis in foods and beverages was discussed. Different sample preparation methods including hydrolyzation and derivatisation of the amino acids were presented alongside different separation and detection techniques.

Amino acids are important organic molecules that can be found in every living cell. The amino acids play an important role in forming proteins, which makes them essential to humans. Human Proteins consist of 20 amino acids, of which nine are essentials and need to be obtained from food. Analysis of amino acids in foodstuff gives important information about the product and can be used for several purposes. In addition to the nutritional value, the amino acid concentration in foods and beverages constitutes the product's flavour, quality, and freshness. This literature study presented several amino acid analyses that have been widely applied to analyse food and beverage samples, and as research continues, even more applications are to be expected. One application is introduced in the experimental part of this study, in which an enzymatic photometric method is used to analyse L-asparagine and L-aspartic acid in foodstuff.

Sample preparation is an important step in amino acid analysis in food and beverage samples. The foodstuff as a matrix can be extremely complex and contain several interfering substances; thus, careful sample preparation is often needed. Successful sample preparation separates amino acids from interfering substances and improves chromatographical separation. The sample preparation consists almost invariably of protein hydrolysis, which is used to free amino acids that are bound into proteins. Common hydrolysis processes, such as acid and alkaline hydrolysis, are widely used. The hydrolysis procedure is selected carefully as none of the common methods can simultaneously hydrolyse the common amino acids. In addition to hydrolysation, different extraction methods are used to extract amino acids in food samples before analysis. Such methods are traditional and vastly used SLE and LLE methods. Newer extraction methods, such as, SPEM extracts analytes more efficiently and with less organic solvent.

Derivatisation of amino acids is used vastly to improve the detection methods sensitivity or chromatographical selectivity. Depending on the analysis method, different derivatization reagents can be employed. Commonly used derivatisation reagents are OPA, PITC, Dansyl chloride, and Methyl chloroformate. Depending on the derivatization reagent, the derivatisation conditions and reaction time vary significantly. OPA derivatisation takes only a couple of minutes, which has been utilised by automating the OPA derivatisation process.

Versatile analysis methods have been developed during the past two decades to analyse amino acids in foodstuff. Traditional and still the most used analysis methods are GC and HPLC coupled with either UV or fluorescence detector. These analysis methods are selective and sensitive but don’t compare to the sensitivity achieved with more developed analysis methods such as UHPLC and mass spectrometry.

# 7. Introduction to experimental study

The background to this experimental study lies in the European Chemical Agency's decision to restrict the use of Triton X-100 chemical. The European Chemical Agency (ECHA) has added the Triton X-100 into the authorization list of substances, included in Annex XIV of REACH (Registration, Evaluation, Authorization, and Restrictions of Chemicals).[49] The REACH regulation aims to protect humans and environments from hazardous chemicals, also referred to as substances of very high concern (SVHC).[50] When a chemical has been placed on the authorization list, the usage of the said substance has to be authorized by ECHA after a sunset date. For Triton X-100, the sunset date was 4.1.2021, and after that, the Triton X-100 has been prohibited without exemption from authorization.[49]

Triton X-100 is used as a non-active detergent in Thermo Fisher's analyser kit for photometric determination of L-asparagine and L-aspartic acid. The aim of this project was to find a suitable replacer for Triton X-100 in L-Asp/L-AspAc test and to perform feasibility tests for the modified method. Criteria for the new method was to have as good or better performance as the current method. The replacing detergent should be cost-efficient and easily available from a reliable producer.

This experimental study was commissioned by the industrial R&D laboratory of Thermo Fisher Scientific Oy in Vantaa during the summer of 2019.

# 8. Theory

## 8.1. Triton X-100

Triton X-100 (2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol[51]) is a non-ionic surfactant, which consists of a phenoxyethanol and trimethylpentane. The structure of Triton X-100 is presented in Figure 10.

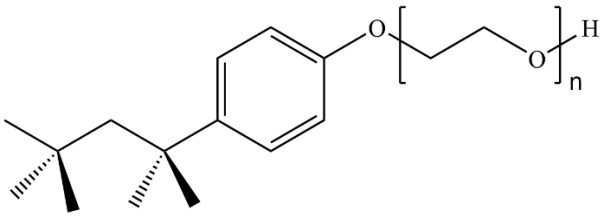


*Figure 10. Structure of Triton X-100.*

As seen from Figure 10, the phenol group of Triton X-100 is connected to a hydrophobic trimethylpentane and a hydrophilic oxyethanol. Triton X-100 is classified as detergent, surfactant and antifoaming agent and can be used, for example, to modify solution's surface tension.[52] When the Triton X-100 concentration in the solution is lower than the critical micelle concentration (CMC), the Triton X-100 reduces foaming and foam stability in solution.[53] Critical micelle concentration represents the concentration where the surfactant molecules start forming micelles.[54] In the L-Asp/L-AspAc test the Triton X-100 reduces the amount of bubbles that form in the cuvettes and thus increases method's precision.

Triton X-100 is classified as corrosive, irritant and environmentally hazardous compound and it is very toxic to aquatic life with long lasting effects (H410).[52] For humans the compound is harmful when swallowed (H302) and can cause skin irritation (H315) and serious eye damage (H318).[52] The compound has also endocrine disrupting properties[51] in environment and because of these reasons Triton X-100 was classified as a substance of very high concern by ECHA. As per ECHAs goals to replace the SVHCs with less dangerous chemicals the Triton X-100 was placed on the ECHAs authorization list.[49]

## 8.2. Enzymatic reactions L-Asp and L-AspAc

The determination of L-asparagine/L-aspartic acid with the Thermo Fisher reagent kit is based on enzymatic reactions and photometric detection. When analysing L-asparagine, enzyme L-asparaginase is used to hydrolyse L-asparagine to L-aspartic acid and ammonia according to the Chemical Equation 1.[55]

*Chemical Equation 1. Hydrolysis of L -asparagine to L -aspartic acid and ammonia*

$$\text{L-Asparagine} + \text{H2O} \xrightarrow{\text{L-Asparaginase}} \text{L-Aspartic Acid} + \text{NH3} \qquad (1)$$

L-aspartic acid and alpha-ketoglutarate are converted into L-glutamic acid and oxaloacetate (Chemical Equation 2). The enzyme glutamate-oxaloacetate transaminase (GOT) in the presence of alpha-ketoglutarate, converts the L-aspartic acid to oxaloacetate.[55]

*Chemical Equation 2. Reaction catalysed by glutamate-oxaloacetate transaminase.*

$$\text{L-Aspartic Acid} + \alpha\text{–Ketoglutarate} \underset{}{\overset{\text{GOT}}{\rightleftharpoons}} \text{Oxaloacetate} + \text{L-Glutamic Acid} \qquad (2)$$

Finally, malate dehydrogenase (MDH) is used to catalyse the reduction of oxaloacetate to L-malic acid in the presence of nicotinamide adenine dinucleotide (NADH) according to the Chemical Equation 3.[55]

*Chemical Equation 3. Malate dehydrogenase catalysed reduction of oxaloacetate through oxidation of NADH.*

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \overset{\text{MDH}}{\rightleftharpoons} \text{L-Malic acid} + \text{NAD}^+ \qquad (3)$$

The concentration of NADH can be observed spectrophotometrically at 340 nm. When analysing the concentration of L-aspartic acid, the Chemical Equations 2 and 3 are used. The amount of oxidised NADH in equation 3, is stoichiometrically equal to the amount of L-aspartic acid in the sample.[55] When analysing the L-asparagine the regent R4 containing the L-asparaginase enzyme

is added and all the Chemical Equations 1-3 are followed through. The concentration of NADH is a sum of L-asparagine and L-aspartic acid concentrations in the sample, thus blanking is performed to subtract the L-aspartic acid concentration from the result.

# 9. Gallery Plus Instrument

Thermo Scientific™ Gallery Plus™ is a discrete analyser, that is designed for photometric analyses including colorimetric, enzymatic and electrochemical analyses.[56] The Galley Plus analyser covers a wide range of different applications, such as tests for analysing food, beverage, water and environmental samples. The analyser is fast, and it allows the simultaneous photometric analysis of different analytes from multiple samples.[56] With the sample capacity of 109, the analyser can analyse up to 350 tests per hour. The cuvettes used in the analyser are small and have a total volume of 300 µl, which leads to small reagent volumes and reagent waste. This results in reduced reagent costs.[56]

Figure 11 presents the Gallery Plus analyser.[56] The portable benchtop analyser is compact, and it is operated through three covers. The main cover is divided into three separate covers, which are used for inserting samples, reagents and cuvettes into analyser. The Gallery Plus has separate containers for ionised water, waste and used cuvettes, these are positioned under the green covers. Thanks to the containers the analyser is truly self-supporting and requires only power supply.[56] This allows the Gallery Plus to be placed and moved easily in the laboratory.

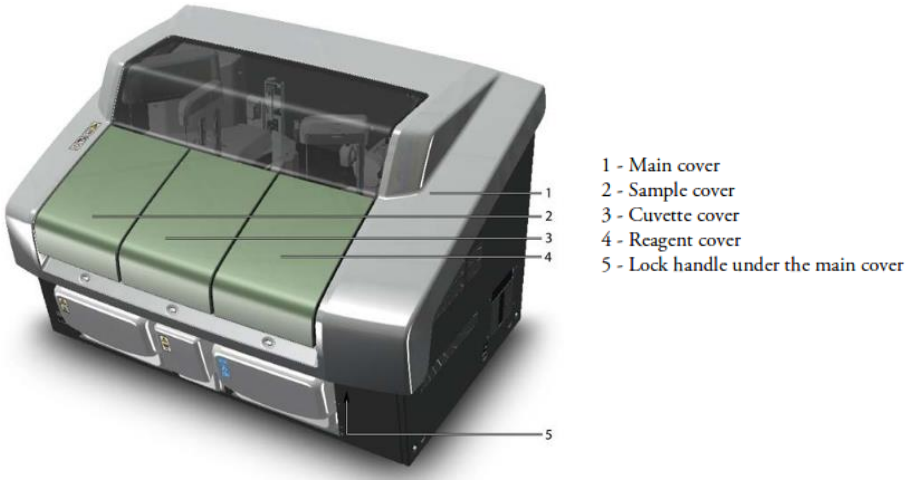


*Figure 11. Gallery Plus analyser. Reprinted with permission from Thermo Fisher Scientific.* [56]

## 9.1. Operational principle

The operation principle of the Gallery Plus analyser is demonstrated in Figure 12.[57] As the Figure 12 illustrates, the cuvettes (1) are inserted into the cuvette loader (2), from where they are loaded in the incubator (3). The incubator rotates and moves each cuvette between the fixed locations for sample and reagent insertion, mixing, and photometric measurement. The samples are placed in a 9-cell sample rack (4), which is inserted into sample disk (5). The reagents (6) provided by Thermo Fisher, are inserted one by one into regent disk (7). The barcode readers (8) read the barcodes from the reagent bottles and sample racks. Reagent (9) and sample dispensers (10) transfer samples and reagents into cuvettes. Mixer (11) is used to homogenize the sample solutions in cuvettes. Finally, the photometric unit (12) measures the absorption from the sample cuvettes. After the whole cuvette row is measured, it is discarded into a cuvette waste bin and replaced by a new cuvette row. The analyser is totally automatized, and it allows insertion of samples, regents and cuvettes during analysis.

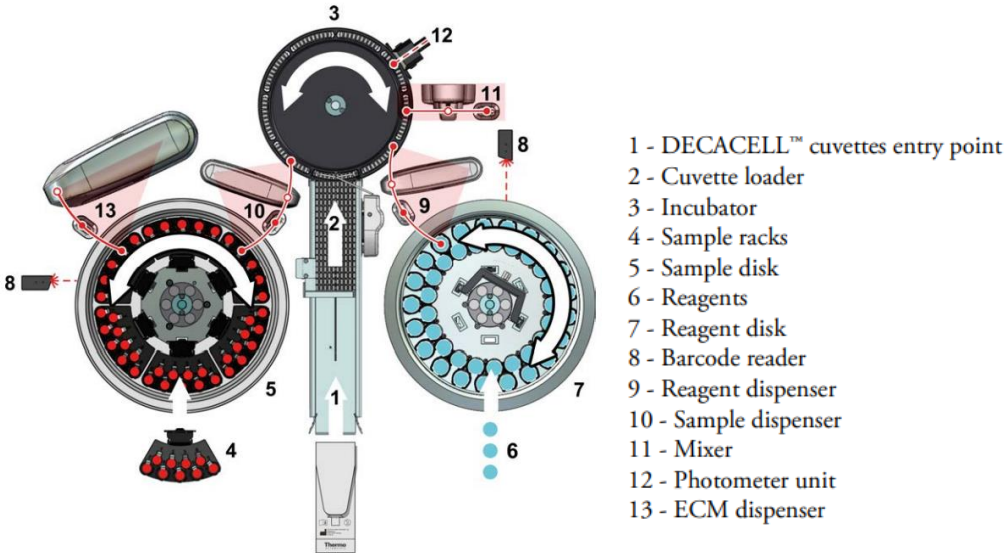


*Figure 12. Operation principle of Gallery Plus analyser. Reprinted with permission form Thermo Fisher Scientific.* [57]

The Gallery Plus has a traditional UV/Vis detector to measure the concentrations of analytes in the samples.[57] The method is based on the Beer – Lambert law, which states that concentration is directly proportional to the absorbance. Figure 13 schematically presents the operation principle of the UV/Vis detector.[57] The light source (1) emits a light beam (2), which has all wavelengths before it is filtered (3). The filtered monochromatic light beam (4) is directed by an optical fiber (5) to a beam splitter (6). The beam splitter divides the light beam into two parts, one light beam becomes a reference beam (7) and is measured by a reference detector (8). The second light beam is a signal beam (10) and travels thorough a sample cuvette that absorbs some of the light. The signal beam is measured with a signal detector (11) and the absorbance is calculated.

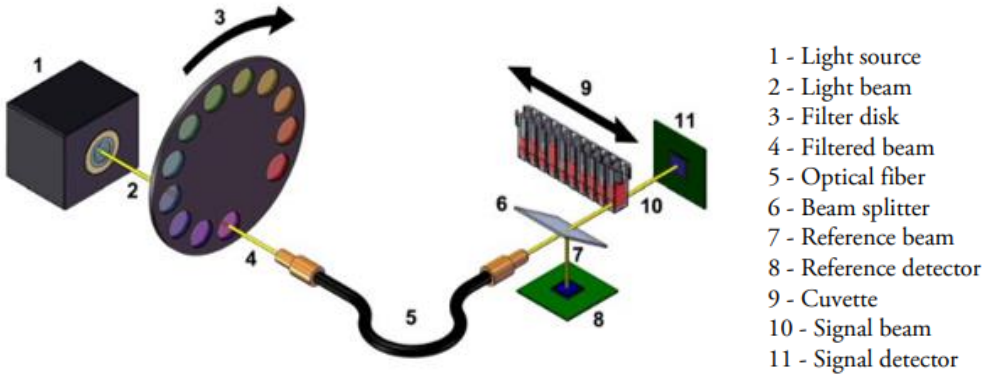


*Figure 13. Operation principle of the UV/Vis detector. Reprinted with permission form Thermo Fisher Scientific.*[57]

The absorbance is calculated from the intensities of detected light beams according to Equation 3.

*Equation 3. Relationship between absorbance and intensities of detected light beams.*

$$A = \log_{10} \frac{I_R}{I_S} \tag{3}$$

Where, A is absorbance, $I_R$ is intensity of reference beam, $I_S$ is intensity of signal beam.

When using main and side wavelength for the measurement, the absorbance of the side wavelength is reduced from the main wavelength's absorbance. In addition, blank's absorbance is also reduced from the absorbance measured from the cuvette. This is done to reduce the absorbance caused by the sample matrix. The response is calculated with Equation 4.

*Equation 4. Response calculated from measured absorbances.*

$$Response = (A_{Main} - A_{Side}) - (A_{BL\_M} - A_{BL\_S}) \quad (4)$$

where the $A_{Main}$ is absorbance of the main wavelength, $A_{Side}$ is the absorbance of the side wavelength, $A_{BL\_M}$ is blank's absorbance of the main wavelength, $A_{BL\_S}$ is the blank's absorbance of the side wavelength.

Based on the response, the sample concentration is calculated by the calibration equation. The type of calibration equation depends on the analyte and is selected for each analyte by fitting the calibration points to the most suitable equation type. The calibration type can be, for example, linear, 2nd order, point-to-point or spline calibration.[57] For L-Asp/L-AspAc the calibration curve is linear and the basic form of the calibration equation (5) is as follows:

*Equation 5. Slope-intercept form of linear equation.*

$$y = ax + b \quad (5)$$

where, a is slope and b is intercept.

The analyser calculates automatically concentration of analysed samples, by placing the measured response into a calibration equation.

# 10. Experimental

## 10.1. Instruments

The Thermo Scientific Gallery Plus analyser was used to analyse L-asparagine/L-aspartic acid concentrations in food samples. The data processing was carried out with Gallery software version 6.0.1. Analytical balance from Sartorius Lab Instruments, model Quintix224-1S was used to prepare the reagents, standard solutions and samples. For controlling the pH, the Thermo Scientific's Orion 3 Star pH benchtop pH-meter was used with Orion -Ross combination pH electrode. An UV-Vis spectrophotometer from Thermo Scientific, model Multiskan GO (software 3.2.1.4) was used to measure the reaction spectrums. An Elix 35 Clinical water purification system from Millipore was used to produce the analytical grade water used throughout the testing. In addition, general laboratory equipment was used including glassware, pipettes, hotplate and magnetic stirrer.

## 10.2. Gallery Applications

### 10.2.1. L-asparagine applications

Thermo Scientific system reagent kit "L-Asparagine/L-Aspartic acid" is used for the determination of L-asparagine in food and beverage samples. The enzymatic test method is based on the enzymatic reactions of malate hydrogenase and L-asparaginase. The method measures the concentration of the NADH, which is indirectly proportional to the concentration of the L-asparagine. The reaction is descending, and the photometric measurement is done with main wavelength of 340 nm and side wavelength of 700 nm. A linear calibration curve is used to calculate the results. The tests measuring range is 20 – 500 mg/l with calibration range of 0 – 100 mg/l. The test flow is as follows: Reagent 1 (R1) and sample (S) are dispensed with extra (X) volume into a cuvette. The cuvette is incubated (inc.) after which reagent 2 (R2) and reagent (R3) are dispensed with extra and water (W) respectively. After second incubation the end-point blank (Bl.) is measured. Finally, reagent 4 (R4) is dispensed with water into the cuvette and after third

incubation the end-point measurement (Meas.) is done with main wavelength of 340 nm and side wavelength of 700 nm.

For the candidate methods 1-3 (C1-C3) new applications were created based on the original L-Asp application. The new applications were otherwise identical to the original L-Asp application, but the reagent R1 was changed to the new modified reagent. In the Table 7 are listed all the applications used for the preliminary testing.

*Table 7. L-asparagine applications used in feasibility tests.*

| **Application name** | **Application** |
|---|---|
| L-Asp | R1 75 µl (+20X), S25 µl (+30X), inc. 120s, R2 20 µl (+10X), R3 35 µl (+15W), inc. 420s, Bl., R4 30 µl (+15W), inc. 420s, Meas. 340 nm (700 nm) |
| L-Asp Kin | R1 75 µl (+20X), S25 µl (+30X), inc. 120s, R2 20 µl (+10X), R3 35 µl (+15W), inc. 420s, Bl., R4 30 µl (+15W), Kinetic: 340 nm (700 nm), 700 s/12 points. |
| L-Asp Blank | R1 75 µl (+20X), S25 µl (+30X), inc. 120s, R2 20 µl (+10X), R3 35 µl (+15W), Kinetic: 340 nm (700 nm), 700 s/12 points. |
| L-Asp_C1 | Same as L-Asp, but R1 is replaced with R1_C1 |
| L-Asp_C2 | Same as L-Asp, but R1 is replaced with R1_C2 |
| L-Asp_C3 | Same as L-Asp, but R1 is replaced with R1_C3 |
| L-Asp Kin_C1 | Same as L-Asp Kin, but R1 is replaced with R1_C1 |
| L-Asp Kin_C2 | Same as L-Asp Kin, but R1 is replaced with R1_C2 |
| L-Asp Kin_C3 | Same as L-Asp Kin, but R1 is replaced with R1_C3 |
| L-Asp Blank_C1 | Same as L-Asp Blank, but R1 is replaced with R1_C1 |
| L-Asp Blank_C2 | Same as L-Asp Blank, but R1 is replaced with R1_C2 |
| L-Asp Blank_C3 | Same as L-Asp Blank, but R1 is replaced with R1_C3 |

For kinetic measurements both reaction kinetics and blank kinetics are measured. Kinetics are measured to ensure that incubation times are adequate. The application for measurement of reaction kinetics has otherwise the same flow as the L-Asp application, but the incubation before the end-point measurement is replaced with kinetic measurement, where the absorbance is measured multiple times within given time interval. Similarly, the application of blank kinetics has a kinetic measurement that replaces the incubation before measuring the blank. The applications of the original blank and kinetic measurements are presented in the Table 7 alongside the modified candidate applications.

### 10.2.1. L-aspartic acid applications

The L-AspAc application is used to determine L-aspartic acids concentration from the food samples. L-AspAc application closely resembles the L-Asp application, the difference is that with L-AspAc application reagent 4 is not used and because of this the dispensed volumes are larger to make up the needed cuvette volume. Applications for the chosen candidate method were created based on the official applications. All the used applications are listed in the Table 8.

*Table 8. L-aspartic acid applications used in feasibility tests.*

| Application name | Application |
|---|---|
| L-AspAc | R1 90 µl (+20X), S30 µl (+60X), inc. 120s, Bl. R2 25 µl (+50X), inc. 420s, R3 45 µl (+20W), inc. 420s, Meas. 340 nm (700 nm) |
| L-AspAc Kin | R1 90 µl (+20X), S30 µl (+60X), inc. 120s, Bl. R2 25 µl (+50X), inc. 420s, R3 45 µl (+20W), Kinetic: 340 nm (700 nm), 700 s/12 points. |
| L-AspAc Blank | R1 90 µl (+20X), S30 µl (+60X), Kinetic: 340 nm (700 nm), 700 s/12 points. |
| L-AspAc_C3 | Same as L-AspAc, but R1 is replaced with R1_C3 |
| L-AspAc Kin_C3 | Same as L-AspAc Kin, but R1 is replaced with R1_C3 |
| L-AspAc Blank_C3 | Same as L-AspAc Blank, but R1 is replaced with R1_C3 |

## 10.3. Reagents and Chemicals

Thermo Scientific reagent kit containing the reagents and calibrator for L-asparagine/L-aspartic acid test were used. The L-Asp test requires four reagents, wash fluid and calibrator whereas the L-AspAc test requires only three reagents. All of these are provided by Thermo Scientific and they are ready to use. The main compositions of the regents, calibrator and washfluid are listed in the Table 9.

*Table 9. Composition of reagents, calibrator and washfluid used for feasibility tests.*

| Reagent | Solution | Concentration |
|---|---|---|
| **L-Asp R1** | Phosphate buffer, pH 7.2 | 250 mmol/l |
| **L-Asp R2** | NADH solution | 6 mmol/l |
| **L-Asp R3** | GOT solution | 30 kU/l |
| **L-Asp R4** | Asparaginase solution | 4 kU/l |
| **Standard** | L-asparagine | 400 mg/l |
| | L-aspartic acid | 200 mg/l |
| **Washfluid** | HCl solution | < 2 % |

The Washfluid is used after the reagent R4 is dispensed into the cuvette to wash the reagent dispenser and reduce carry over. Otherwise, the dispensers are rinsed with water between injections.

In addition to reagents, several chemicals were used. Chemicals L-asparagine and L-aspartic acid were used to prepare stock solutions and quality control (QC) samples. In potato and asparagus sample preparation, Carrez I and II solutions and 1-octanol were used. The reagents, standard solutions and samples were stored in refrigerators and the chemicals were stored in chemical cupboards. The reagents, calibrator and chemicals used in the feasibility tests are listed in the Table 10.

*Table 10. Reagents, calibrator and chemicals used for feasibility tests.*

| Reagent | Ref | Lot | Exp | Manufacturer |
|---|---|---|---|---|
| L-Asp R1 | 984319 | R004 | 31.12.2019 | Thermo Fisher Scientific (TFS) |
| L-Asp R2 | 984319 | R004 | 31.12.2019 | TFS |
| L-Asp R3 | 984319 | R004 | 31.12.2019 | TFS |
| L-Asp R4 | 984319 | R004 | 31.12.2019 | TFS |
| Standard | 984319 | R197 | 31.1.2020 | TFS |
| Washfluid | 984841 | PA43 | 31.8.2020 | TFS |
| L-asparagine | A0884-25G | SLBM5864V | 02.2020 | Sigma-Aldrich |
| L-aspartic acid | A8949-25G | BCBL8875V | 06.2022 | Sigma-Aldrich |
| Carrez I | 03323.2000 | 19020037 | 5.2022 | Bernd Kraft |
| Carrez II | 03324.2000 | 19020095 | 5.2021 | Bernd Kraft |
| 1-Octanol | A15977 | 10206490 | 6.2021 | Alfa Aesar |

## 10.4. Preparation of solutions

### 10.4.1. Standard stock solutions and quality control samples

The standard stock solution is used to prepare quality control samples. The stock solutions for L-asparagine analysis were prepared weekly by weighing 40 mg of L-asparagine with an analysis balance. The weighed L-asparagine was dissolved with deionized water in a volumetric flask of 100 ml. The concentration of the stock solution was 400 mg/l. The stock solution was stored in a refrigerator and used within five days. Fresh quality control samples were prepared daily from the stock solution. The high QC sample was prepared by diluting the stock solution 1:5 with deionized water. The concentration of the high QC sample was 80 mg/l. The low QC sample was diluted from the high QC samples with deionized water in a 1:2 ratio, resulting in a concentration of 40 mg/l. The dilutions were done with semiautomatic pipettes and volumetric flasks. A sample preparation protocol was filled and saved for every prepared stock solution. The protocol calculates the accurate concentrations of the solutions and gives the acceptance limits for QC samples.

The standard stock solution for L-aspartic acid was prepared in the same manner as the stock solution for L-asparagine. The stock solution was stored and used within five days. The quality control samples were prepared from the stock solution by dilution. The concentration of the high QC was 80 mg/l, and the low QC sample 40 mg/l. Fresh QC samples were prepared on every analysis day.

### 10.4.2. R1 candidate reagents

Three new R1 reagents were prepared in which Triton X-100 detergent was replaced with another detergent. The three different detergent candidates for feasibility testing were selected from the company's list of recommendations. The selection was based on detergent's chemical properties, C&L (classification and labelling) notifications, CMR (Carcinogenic, mutagenic, reprotoxic) classifications, SVHC potential, and possible hazards during the production or in waste management. From all the possible candidates three were found to be suitable with acceptable price and availability in the market.

The three candidate R1 reagents are referred hereafter as Candidate 1 (R1_C1), Candidate 2 (R1_C2) and Candidate 3 (R1_C3). The candidate R1 reagents were prepared in an exact same manner as the original R1 solution. The Triton X-100 detergent was replaced with a new candidate so that the critical micelle concentration of the new reagents was maintained at the same level as the original reagent.

## 10.5. Samples and sample preparation

### 10.5.1. Potato and asparagus samples

The potato and asparagus samples were prepared in the same manner. The vegetables were first homogenized, the potatoes were peeled and grated, and the asparaguses were grated. A grater with a hole diameter of 3 mm was used. 50 g of potato and asparagus smash was weighed and placed in separate beakers, respectively. In each beaker, 250 ml of deionized water was added, and the mixture was stirred with a magnetic stirrer for 2 minutes. After stirring, both mixtures were filtered with filter paper and funnel. Filter papers were changed multiple times to reduce filtering time. The potato extract was brown and clear, as the asparagus extract was green and clear.
40 ml of potato and asparagus extract was pipetted with a precision pipette and transferred into 250 volumetric flasks, respectively. 50 ml of deionized water, 2 ml of Carrez I and 2 ml Carrez II solutions were added in both volumetric flasks. The solution was mixed slightly between the additions. The colours of both solutions changed; the potato sample changed from brownish to gray, and the asparagus solution changed from vibrant green to cloudy green. Lastly, three drops of octanol were added to the solutions to remove bubbles. The volumetric flasks were filled up to the marks with deionized water, and the solutions were filtered. After the filtration, both solutions were clear and colourless, and they were stored in a refrigerator.

Several potato samples and asparagus samples were prepared during the testing period. A list of prepared samples with comments is listed in Table 11. The Table also separates whether the sample was used for L-Asp or L-AspAc testing. The potato samples 1-3 were used in the preliminary testing phase, where the suitability of the candidate methods were compared. After one of the candidate methods was chosen, the method's performance was also tested with the L-AspAc test. For this, "Potato 4" sample was prepared as the previously prepared samples were no longer fresh. The asparagus samples were prepared to test the chosen candidate's performance for both L-Asp and L-AspAc methods with a different matrix.

*Table 11. Samples prepared for L-Asp/L-AspAc feasibility tests.*

| Sample | Comment | Tested with L-Asp/L-AspAc |
|---|---|---|
| **Potato 1** | Prepared in parallel with "Potato 2" from the same potato mash. During preparation, the carrez solutions were added in incorrect order. | L-Asp |
| **Potato 2** | Prepared in parallel with "Potato 1" sample from same potato mash. During preparation, the carrez solutions were added in incorrect order. | L-Asp |
| **Potato 3** | No additional comments | L-Asp |
| **Potato 4** | No additional comments | L-AspAc |
| **Asparagus 1** | Sample was not used for testing | - |
| **Asparagus 2** | Prepared from the Asparagus 1 sample by diluting with deionized water with ratio 1:2. | L-Asp |
| **Asparagus 3** | Prepared a more concentrated asparagus sample by using the same asparagus extract as in "Asparagus 1" sample. The undiluted extract was treated with Carrez and octanol in a same manner as "Asparagus 1". | L-AspAc |

### 10.5.2. Spiked samples

The spiked samples were prepared by spiking the potato and asparagus samples with either L-Asp or L-AspAc stock solutions. The wanted spike concentration was usually 20 mg/l (c2), and for each spiked sample, the needed volume of stock solution was calculated from the concentration of the stock solution (c1) and the final volume of the sample (V2). The spike addition needed to be under 5 percent in volume of the total sample volume. Below is an example calculation for the "Potato 1 spike" sample, where the stock solutions concentration is 1000 mg/l, wanted spike concentration is 20 mg/l and the sample's volume is 20 ml.

$$V_1 = \frac{c_2 V_2}{c_1} = \frac{20\ mg/l * 20\ ml}{1000\ mg/l} = 0.4\ ml$$

The spike samples were prepared by pipetting the calculated amount of spike solution into a volumetric flask and filling the flask up to the mark with potato or asparagus sample. In the Table 12 are gathered the information about how each L-asparagine spike sample was prepared. From the table it is noticeable, that even though stock solution with different concentrations were used, each sample's spike concentration was 20 mg/l.

*Table 12. L-asparagine spike samples*

| Sample | L-Asp stock solution concentration (mg/l) | Stock solution volume (ml) | Total sample volume (ml) | Spike concentration (mg/l) |
|---|---|---|---|---|
| **Potato 1 spike** | 1000 | 0.4 | 20 | 20 |
| **Potato 3 spike** | 1000 | 0.4 | 20 | 20 |
| **Asparagus 2 spike** | 400 | 0.5 | 10 | 20 |

The L-AspAc spiked samples were prepared in the same manner as L-Asp spike samples. The L-aspartic acid concentration in the potato samples was lower than the L-asparagine concentration and therefore higher spike concentrations were used in the "Potato 4 spike" sample, as shown in Table 13. The L-AspAc spike samples were prepared according to the Table 13.

*Table 13. L-aspartic acid spike samples*

| Sample | L-AspAc stock solution concentration (mg/l) | Stock solution volume (ml) | Total sample volume (ml) | Spike concentration (mg/l) |
|---|---|---|---|---|
| **Potato 4 spike** | 1000 | 0.5 | 10 | 50 |
| **Asparagus 3 spike** | 400 | 0.5 | 10 | 20 |

### 10.5.3. Linearity samples

For linearity tests, L-asparagine stock solution with concentration of 1000 mg/l and an intermediate solution with concentration of 500 mg/l was prepared. The samples for linearity test were prepared by diluting the stock solution or the intermediate solution to give following sample concentrations: 0, 18, 20, 40, 60 ,90, 100, 110, 200, 300, 400, 500, 550 mg/l. A calculation table was used to calculate the volume of stock solution added into the linearity samples. When the total sample volume is 4 ml, and the concentration of the sample is known, the needed amount of stock solution is calculated with the Equation 6. The amount of stock solution required for samples with a final volume of 4 ml and a concentration of 550 mg/l is calculated below.

*Equation 6. Dilution equation for solutions.*

$$c_1 V_1 = c_2 V_2 \tag{6}$$

$$V_1 = \frac{c_2 V_2}{c_1} = \frac{550\ mg/l * 4\ ml}{1000\ mg/l} = 2.2\ ml$$

where $c_1$ is concentration of the stock solution, $V_1$ is volume of the stock solution, $c_2$ is wanted sample concentration and $V_2$ is volume of the final sample.

The linearity samples were prepared by first pipetting 4 ml deionized water into every test tube. The wanted sample concentration was achieved by first removing the same amount of water from the test tube, which was the calculated volume of the required stock solution ($V_1$). After removal of water, the same amount of stock solution was pipetted into the sample solution. For example, when preparing the "550 mg/l" sample 2.2 ml of water was first pipetted from the test tube, and after that 2.2 ml of stock solution was inserted into the same test tube. This pipetting technique reduces the possibility of pipetting errors, because only one pipetting volume is required for each linearity sample. The samples were mixed before analysis. Table 14 shows the calculated stock solution volumes ($V_1$) for each linearity sample. More diluted samples were prepared from the intermediate solution. The samples were prepared according to the volumes in Table 14.

*Table 14. Calculated results for the preparation of L-asparagine linearity samples*

Stock solution conc. ($c_1$): 1000 mg/l
Intermediate conc. ($c_1$): 500 mg/l
Sample volume ($V_2$): 4 ml

| **Sample conc. ($c_2$) (mg/l)** | **Stock vol. ($V_1$) (ml)** |
|---|---|
| 550 | 2.2 |
| 500 | 2 |
| 400 | 1.6 |
| 300 | 1.2 |
| 200 | 0.8 |

| **Sample conc. ($c_2$) (mg/l)** | **Intermediate vol.($V_1$) (ml)** |
|---|---|
| 110 | 0.88 |
| 100 | 0.8 |
| 90 | 0.72 |
| 60 | 0.48 |
| 40 | 0.32 |
| 20 | 0.16 |
| 18 | 0.14 |
| 0 | 0 |

The L-aspartic acid linearity samples were prepared from a stock solution with a concentration of 1004 mg/l. Since the testing was performed after the suitable candidate method was selected and the linearity of the method was already tested with L-Asp method, the linearity for L-AspAc method was tested lightly. This was accomplished by measuring linearity only with samples that were outside the test limits. Samples were prepared according to Table 15.

*Table 15. Calculated results for the preparation of L-aspartic acid linearity samples*

L-AspAc Stock solution conc. ($c_1$): 1004 mg/l
Sample volume ($V_2$): 4 ml

| **Sample conc. ($c_2$) (mg/l)** | **Stock vol. ($V_1$) (ml)** |
|---|---|
| 18 | 0.072 |
| 90 | 0.359 |
| 110 | 0.438 |
| 550 | 2.191 |

## 10.6. Feasibility tests

The feasibility tests were conducted for the three candidate methods with modified R1 reagents (R1_C1-3) and for the original L-asparagine method. The criteria for candidate methods were to have as good or better performance as the original L-Asp test. The performance was evaluated by testing preliminary precision, accuracy, and linearity. Also, a comparison between the calibration, kinetics, and reaction spectrum of the candidate methods and the original method was tested.

The preliminary tests with three different candidates were conducted only with the L-asparagine test. This is because the L-asparagine test has one reaction step more than the L-aspartic acid test and it is assumed to have a bigger deviation. After an appropriate candidate was chosen, the performance was also tested for the L-aspartic acid test.

**Calibration**

The analyser was calibrated every day before analysis. The calibration curve was to be in accordance with the previous calibrations, and the results of high and low QC samples (n=3) had to be within given limits. A ready-to-use calibrator and freshly prepared QC samples were used for calibration.

**Precision**

The preliminary precision was determined on QC40, QC80, Potato 1, Potato 2, Potato 3, and Asparagus 2 samples for the L-Asp test. Ten replications (n=10) were run from each sample without interruptions. For the L-AspAc test, the preliminary precision was determined with QC40, QC80, Potato 4, Potato 4 spike, and Asparagus 3 samples.

**Accuracy**

The preliminary accuracy was determined with QC40 and QC80 samples by analysing three (n=3) replicates from each sample. The accuracy run was conducted on three different days. The accuracy of each method was also assured when calibrating the methods and running the QC samples.

**Linearity**

Linearity of the three candidate methods were determined by examining the correlation of the analytes measured concentration and theoretical value. Linearity was determined for the measuring range (± 10 %). The measuring range includes a primary test range (20 – 100 mg/l) and a secondary test range (100 – 500 mg/l). The linearity of both test ranges was tested with series of samples that exceeded the test ranges by 10 %.

Linearity of L-Asp test was determined with three candidate methods using L-asparagine samples at 12 different concentrations. Linearity of L-aspartic acids test was determined for the selected candidate method and L-aspartic acid samples at four different concentrations were used for testing.

**Kinetics**

The kinetic measurements include measurement of reaction and blank kinetics. Both kinetic measurements were done for original L-Asp test and for three candidate methods. Similarly, the kinetic measurements were done for L-AspAc test and for the chosen L-AspAc candidate method. The reaction and blank kinetics were measured with separate applications with Gallery analyser.

**Spectral comparison**

The spectrum of the reaction was measured with a spectrophotometer. The measurement was performed for L-Asp test and candidate methods as well as for the L-AspAc test and the chosen candidate method. The spectrum of the original R1 reagent and the three candidate reagents (R1_C1-3) were also measured. The reactions were done in 4 ml by following the original applications workflow. The volumes of the application flow were multiplied with 7 to increase the total volume which was needed for the 4 ml cuvette. The program with each application step was recorded for the spectrophotometer. The incubation times were the same as in original applications.

# 11. Results and discussion

The preliminary tests were conducted parallel on three candidates for the L-Asp test. Based on the preliminary results, one of the candidates was chosen to be the Triton X replacer, and the feasibility testing was continued only with the selected candidate. In the following chapters, the results of the L-Asp preliminary tests conducted with three candidate methods are presented. These results are presented in conjunction with the L-AspAc results, in which only selected candidate C3 was tested. The reasons for selecting the C3 candidate to replace the original method are discussed after the results are presented.

The preliminary test results revealed that any of the three candidates could be chosen to replace the current R1 reagent. A suitable candidate's criteria were to have the same, if not better, performance as the current reagent. The criteria for acceptable performance are listed in Table 16. In addition, the candidate should not have significant cost effects on the product, and it should be easily acquirable.

*Table 16. Criteria for candidate methods*

| **Subject** | **Criteria** |
|---|---|
| QC precision (n=10) | CV% ≤ 5% |
| Sample precision (within run) | Should: CV % ≤ 2.9%<br>Must: CV % ≤ 3 % |
| QC accuracy | Low QC ± 15%<br>High QC ± 10% |
| Spike sample accuracy | ± 15% |

In addition to the abovementioned criteria, the candidate tests must have the same test flow and parameter as the original test. That is to say that the tests' measurement wavelengths, incubation time, calibration range, measuring range, and secondary dilution are to be identical.

## 11.1. Calibration

Calibration was done before analysis and obtained calibration curve was compared with the previous calibration. If the calibration showed no errors and was comparable to the previous calibration, the calibration was accepted. The calibration curve is obtained by plotting the known concentration of the calibration solution against the measured responses. Figure 14 shows the calibration curves and equations for the current and three candidate L-Asp tests.

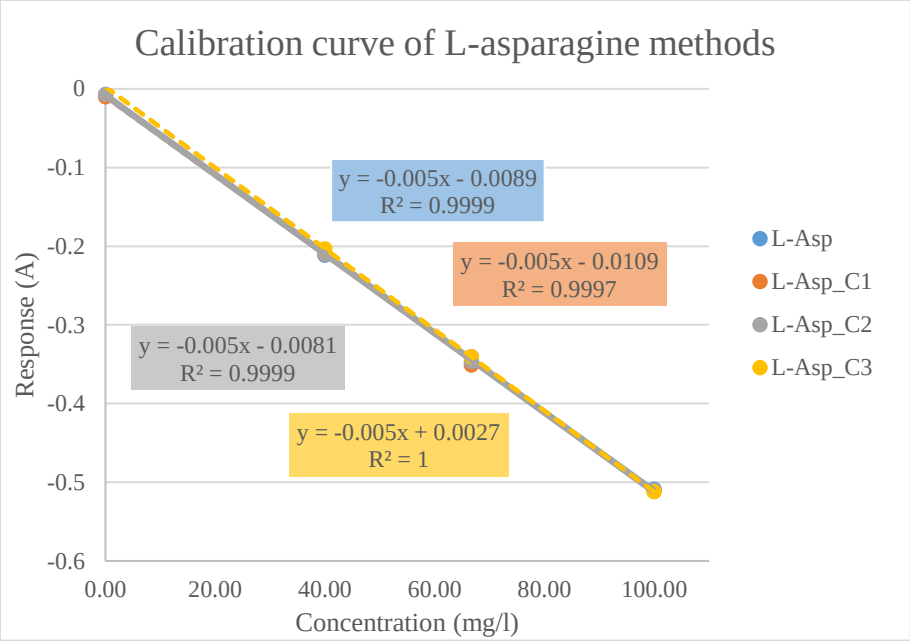


*Figure 14. Calibration curves of L-asparagine methods.*

As can be seen from the calibration graph, all candidate methods have a similar calibration curve as the original L-Asp method. To compare the fit of calibration curves, the concentrations are calculated by placing the measured response in the calibration equation. The difference between the known and calculated concentration is called a residual. The $R^2$ values are squared residuals that represent the difference between the expected and the calculated concentration. The smaller the residual is, the better the calibration curve represents the dataset. In Figure 15 are presented the residuals calculated for each data point of the calibration curve for all four methods.

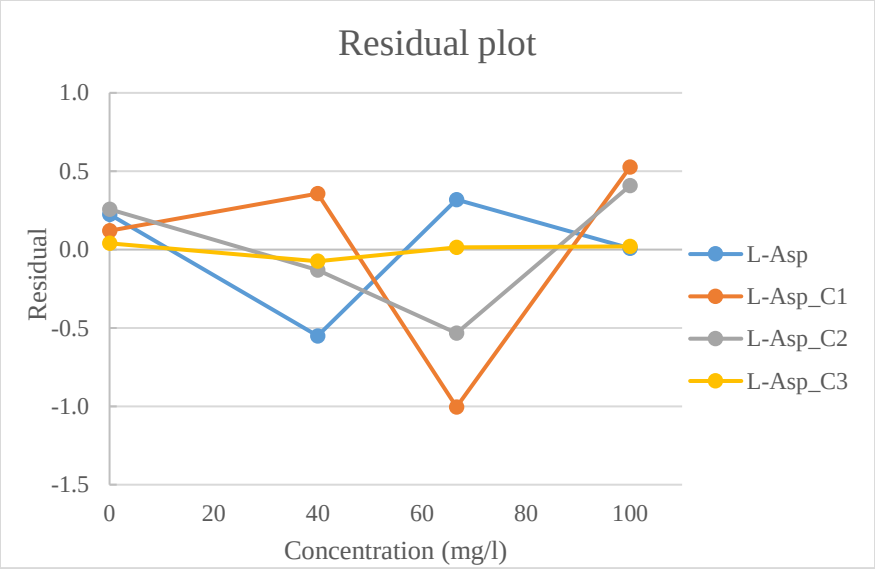


*Figure 15. Calculated residuals from calibration curves of L-Asp methods*

It is clear from Figure 15 that although no method differs significantly from the original L-Asp method, the method L-Asp_C3 has the smallest residuals and is clearly better than the original method. The L-Asp_C1 has the biggest residuals and lowest R2 value, which can be seen from Figure 14. Based on the calibration results, all the new methods had a good correlation with the original method and could be used as a replacing method. Nevertheless, the best calibration curve was obtained with test L-Asp_C3, and it surpassed the original methods' calibration curve.

For L-aspartic acid, the calibration curves were compared between the L-AspAc and L-AspAc_C3 methods. The calibration curves with calibration equations and squared residuals are presented in Figure 16.

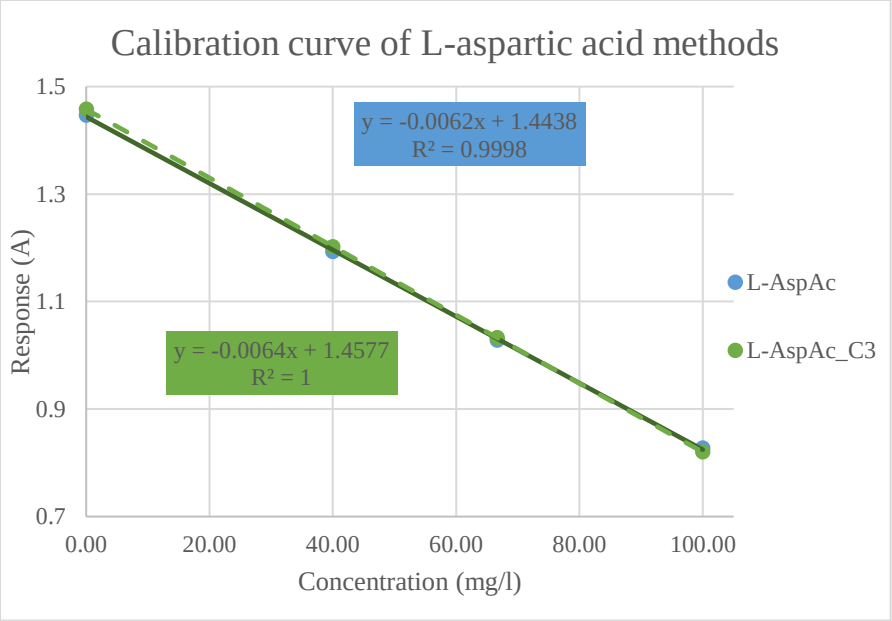


*Figure 16. Calibration curves of L-aspartic acid methods.*

Figure 16 shows that the L-AspAc_C3 calibration curve is very similar to the original method's calibration curve. The R2 value for the L-AspAc_C3 method is also slightly better than for the original L-AspAc method.

## 11.2. Precision

The preliminary precision of the three candidates was determined alongside the current method L-Asp. Testing was done in series of ten replicates for the following samples: QC40, QC80, Potato 1, Potato 2, and Potato 3. The series of replications were tested without interruptions. Table 17 lists the results of precision series with average, standard deviation (SD), and coefficient of variation (CV). After the C3 was chosen to be the replacing method, the precision was also tested with a different sample matrix, asparagus.

*Table 17. Precision results for L-Asp methods.*

| Sample (N=10) | Test | Average (mg/l) | SD | CV% |
|---|---|---|---|---|
| QC40 | L-Asp | 41.8 | 1.0 | 2.3% |
| | L-Asp_C1 | 41.2 | 0.5 | 1.3% |
| | L-Asp_C2 | 41.6 | 0.4 | 1.0% |
| | L-Asp_C3 | 41.7 | 0.7 | 1.7% |
| QC80 | L-Asp | 84.0 | 0.4 | 0.5% |
| | L-Asp_C1 | 83.3 | 0.5 | 0.6% |
| | L-Asp_C2 | 83.5 | 0.4 | 0.5% |
| | L-Asp_C3 | 81.7 | 0.6 | 0.8% |
| Potato 1 | L-Asp | 75.8 | 0.6 | 0.7% |
| | L-Asp_C1 | 75.5 | 0.6 | 0.8% |
| | L-Asp_C2 | 75.7 | 0.4 | 0.5% |
| | L-Asp_C3 | 75.2 | 0.9 | 1.2% |
| Potato 2 | L-Asp | 75.8 | 0.4 | 0.5% |
| | L-Asp_C1 | 76.6 | 0.2 | 0.3% |
| | L-Asp_C2 | 76.6 | 0.4 | 0.5% |
| | L-Asp_C3 | 76.7 | 0.5 | 0.6% |
| Potato 3 | L-Asp | 29.9 | 0.2 | 0.7% |
| | L-Asp_C1 | 29.7 | 0.3 | 1.0% |
| | L-Asp_C2 | 30.3 | 0.4 | 1.3% |
| | L-Asp_C3 | 31.2 | 0.3 | 1.1% |
| Asparagus 2 | L-Asp | 57.45 | 0.27 | 0.47% |
| | L-Asp_C3 | 56.89 | 0.51 | 0.89% |

The preliminary precision results show that all candidates have a very good correlation with the original L-Asp method. As stated in Table 16, the precision criteria for QC samples (n = 10) is CV% ≤ 5 %. All the candidate methods meet this criterion, and in the case of QC40 results, all the candidates have better CV% than the original method. The results of potato and asparagus samples are good and meet the criteria of having a variation coefficient less or equal to 2.9%. It's apparent that the coefficients of variation and standard deviations are well below the acceptance criteria and that there is little variation between methods. The graph in Figure 17 illustrates the means of coefficients of variation and standard deviations calculated from Table 17 results.

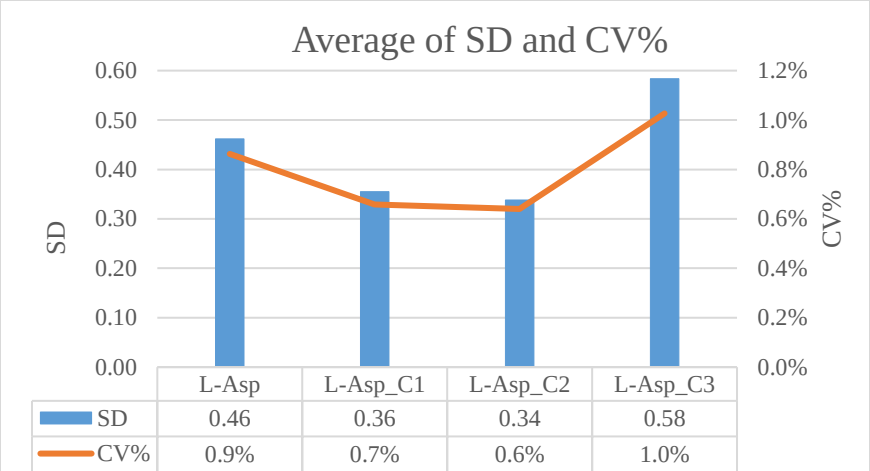


*Figure 17. Averages of the precisison results for L-Asp methods.*

The differences in Figure 17 demonstrate that L-Asp_C1 and L-Asp_C2 have better CV% and precision than the original method L-Asp. Although the L-Asp_C3 method has the highest CV% and SD values, the differences between methods are small, and all methods clearly have adequate performance for precision. Looking more closely at one sample run, the within-run precision is observed. The results of the QC80 sample analysed in ten replicates by different methods, are presented below in Figure 18. The graph illustrates that all the methods produce similar results and that natural variation from sample to sample occurs in all methods.

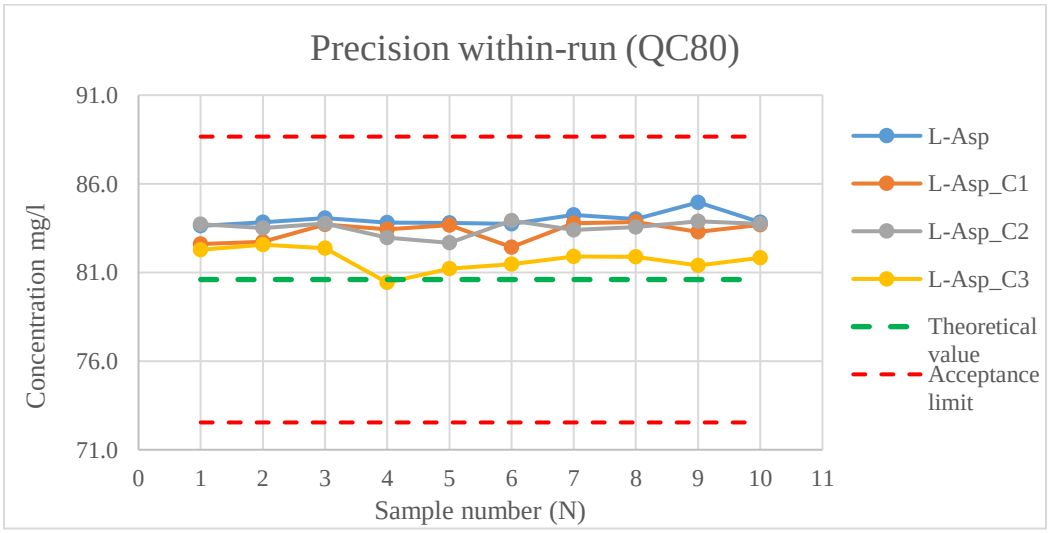


*Figure 18. Precision results of QC80 samples for L-Asp methods. The red dashed lines represent the acceptance limit of the results, while the green dashed line represents the theoretical value of the QC80 sample.*

Figure 18 also illustrates the methods' accuracy. The closer the results are to the theoretical value, the more accurate the method is. According to Table 16, the accuracy criterion for the high QC sample is ± 10%, which is presents in red dashed lines in Figure 18. The results in Figure 18 demonstrate that, on average, all the candidate methods have better accuracy than the original L-Asp test. The results of the L-Asp_C3 test are clearly the closest to the theoretical value. The results indicate that while the L-Asp-C3 test has slightly worse precision than the other tests, the method's accuracy is better than average.

For L-aspartic acid, the precision was asserted from QC samples as well as from potato and asparagus samples. Results from L-AspAc and L-AspAc_C3 methods were compared and the obtained results are gathered in Table 18. As seen from Table 18, the QC sample results are higher for L-AspAc_C3, especially for sample QC40. Results are nonetheless within a criterion, and interestingly the differences between methods are considerably smaller in food samples. Food sample results are almost identical between the two methods, even Potato 4 results. Table 18 shows that the Potato 4 sample concentration is under the test limit (< 20 mg/l), which explains the high CV% values. Both methods behave similarly under the test limit, which is valuable information even though it is not strictly necessary. The potato sample's precision was tested with a spiked potato sample, which concentration was adjusted into measuring range.

*Table 18. Precision results for L-AspAc methods.*

| Sample (N=10) | Test | Average (mg/l) | SD | CV% |
|---|---|---|---|---|
| QC40 | L-AspAc | 38.77 | 0.377 | 0.97% |
| | L-AspAc _C3 | 41.94 | 0.959 | 2.29% |
| QC80 | L-AspAc | 80.29 | 0.595 | 0.74% |
| | L-AspAc _C3 | 81.52 | 0.880 | 1.08% |
| Potato 4 | L-AspAc | 15.89 | 0.554 | 3.49% |
| | L-AspAc _C3 | 19.08 | 0.663 | 3.48% |
| Potato 4 spike | L-AspAc | 67.50 | 1.103 | 1.63% |
| | L-AspAc _C3 | 69.33 | 1.152 | 1.66% |
| Asparagus 3 | L-AspAc | 55.74 | 0.405 | 0.73% |
| | L-AspAc _C3 | 56.74 | 0.322 | 0.57% |

## 11.3. Accuracy

The preliminary accuracy was determined for the original L-Asp method alongside the three candidate methods L-Asp_C1-C3. Accuracy indicates how close the measured result is from the sample's expected value; the closer the result is, the better the method's accuracy. Because accuracy tests are performed on samples of known concentration, quality control samples were used to measure L-Asp methods' accuracy. The test was performed in three days and samples were analysed in three replicates. Table 19 presents the obtained accuracy results with standard deviations and coefficients of variation. In addition to quality controls, Table 19 contains the accuracy results of the spiked potato samples.

*Table 19. Accuracy results for L-Asp methods.*

| Sample (N=3) | Test | Average (mg/l) | SD | CV% | Accuracy % |
|---|---|---|---|---|---|
| **QC40** | L-Asp | 40.79 | 0.86 | 2.1% | 1.0% |
| run 1/3 | L-Asp_C1 | 41.42 | 0.88 | 2.1% | 2.5% |
| | L-Asp_C2 | 41.79 | 0.29 | 0.7% | 3.4% |
| | L-Asp_C3 | 42.04 | 0.21 | 0.5% | 4.1% |
| **QC40** | L-Asp | 34.98 | 0.41 | 1.2% | -13.4% |
| run 2/3 | L-Asp_C1 | 35.00 | 0.13 | 0.4% | -13.4% |
| | L-Asp_C2 | 35.15 | 0.21 | 0.6% | -13.0% |
| | L-Asp_C3 | 35.44 | 0.66 | 1.9% | -12.3% |
| **QC40** | L-Asp | 42.09 | 0.32 | 0.8% | 4.2% |
| run 3/3 | L-Asp_C1 | 42.23 | 0.56 | 1.3% | 4.5% |
| | L-Asp_C2 | 42.17 | 0.59 | 1.4% | 4.4% |
| | L-Asp_C3 | 42.43 | 0.82 | 1.9% | 5.0% |
| **QC80** | L-Asp | 82.26 | 1.67 | 2.0% | 1.8% |
| run 1/3 | L-Asp_C1 | 82.14 | 0.44 | 0.5% | 1.7% |
| | L-Asp_C2 | 82.05 | 0.23 | 0.3% | 1.5% |
| | L-Asp_C3 | 81.48 | 0.77 | 0.9% | 0.8% |
| **QC80** | L-Asp | 83.60 | 0.14 | 0.2% | 3.5% |
| run 2/3 | L-Asp_C1 | 83.60 | 0.13 | 0.2% | 3.5% |
| | L-Asp_C2 | 83.31 | 0.15 | 0.2% | 3.1% |
| | L-Asp_C3 | 83.15 | 0.66 | 0.8% | 2.9% |
| **QC80** | L-Asp | 83.48 | 0.25 | 0.3% | 3.3% |
| run 3/3 | L-Asp_C1 | 83.71 | 0.18 | 0.2% | 3.6% |
| | L-Asp_C2 | 83.47 | 0.47 | 0.6% | 3.3% |
| | L-Asp_C3 | 83.65 | 0.64 | 0.8% | 3.5% |

*Table 19 continues*

| Sample (N=3) | Test | Average (mg/l) | SD | CV% | Accuracy % |
|---|---|---|---|---|---|
| **Potato 1 spike** | L-Asp | 95.24 | 0.14 | 0.2% | -0.5% |
| | L-Asp_C1 | 94.95 | 0.09 | 0.1% | -1.2% |
| | L-Asp_C2 | 94.95 | 0.21 | 0.2% | -0.7% |
| | L-Asp_C3 | 95.17 | 0.13 | 0.1% | -0.5% |
| **Potato 3 spike** | L-Asp | 49.67 | 0.40 | 0.8% | -0.5% |
| | L-Asp_C1 | 49.87 | 0.19 | 0.4% | 0.3% |
| | L-Asp_C2 | 50.26 | 0.06 | 0.1% | -0.1% |
| | L-Asp_C3 | 50.9 | 0.19 | 0.4% | -0.5% |

The results meet the given criteria, i.e., the accuracy for QC40 and spike samples is ± 15%, and that of the QC80 samples is ± 10%. Excluding the results of QC40 run 2/3, all the results are clearly within the acceptance limits. This is demonstrated in Figure 19, where the accuracy results are presented alongside each other.

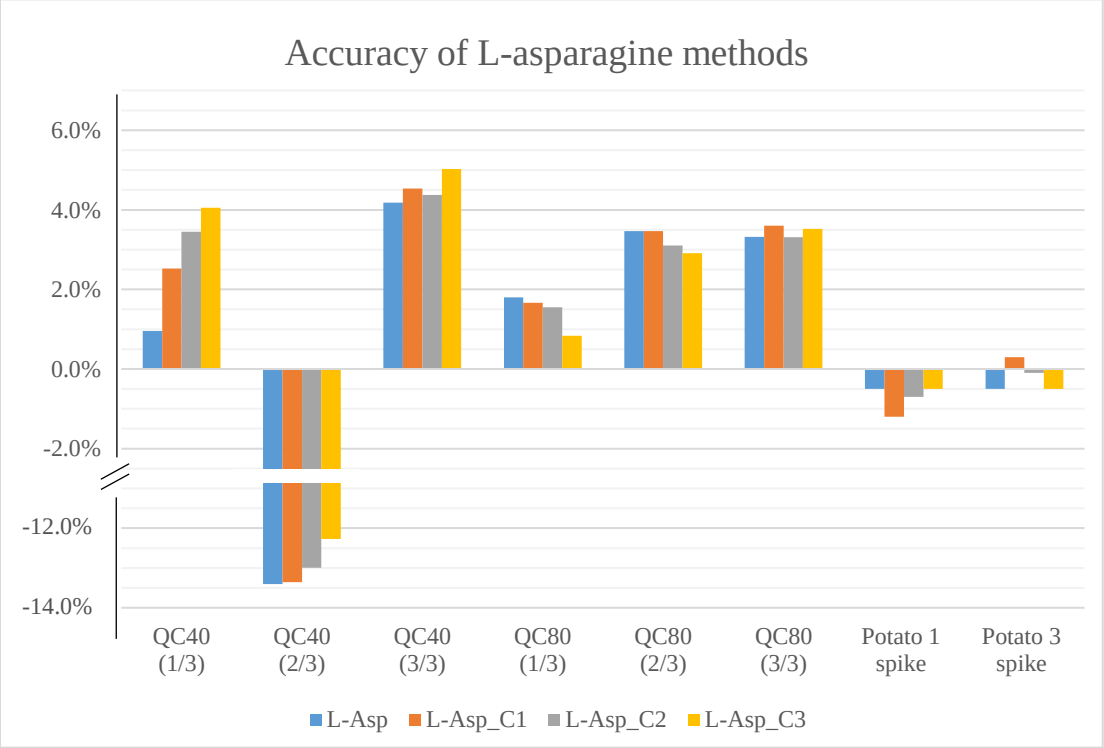


*Figure 19. Accuracy results of L-asparagine methods. The y-axis is truncated between -3% - -11%.*

On average, the candidate methods' accuracy is as good as, if not better, than the accuracy of the original L-Asp method. No candidate method differs considerably from others in terms of accuracy. Daily variation instead is considerable, especially for the QC40 sample, whereas the QC80 results are more consistent. Figure 19 illustrates that the accuracy is substantially better for spiked potato samples than for quality controls. This indicates that a potential matrix effect has little effect on the results and confirms that sample preparation steps have been sufficient.

The accuracy was also tested for L-aspartic acid methods. The results obtained by L-AspAc and L-AspAc_C3 methods for the three different days are shown in Table 20. The accuracy results of the candidate method are clearly better than the results of the original method. The results of both methods meet the criterion and are in line with L-asparagine results.

*Table 20. Accuracy results for L-AspAc methods.*

| Sample (N=3) | Test | Average (mg/l) | SD | CV% | Accuracy % |
|---|---|---|---|---|---|
| QC40 | L-AspAc | 37.86 | 0.93 | 2.5% | -5.8% |
| | | 38.35 | 0.57 | 1.5% | -4.6% |
| | | 39.57 | 0.77 | 1.9% | -1.1% |
| QC40 | L-AspAc_C3 | 39.45 | 0.10 | 0.2% | -1.9% |
| | | 40.23 | 0.17 | 0.4% | 0.1% |
| | | 39.42 | 0.20 | 0.5% | -1.5% |
| QC80 | L-AspAc | 78.48 | 0.52 | 0.7% | -2.4% |
| | | 79.43 | 0.24 | 0.3% | -1.2% |
| | | 80.74 | 0.63 | 0.8% | 0.9% |
| QC80 | L-AspAc_C3 | 78.55 | 0.59 | 0.8% | -2.3% |
| | | 79.40 | 0.19 | 0.2% | -1.2% |
| | | 79.97 | 0.20 | 0.3% | 0.0% |
| Potato 4 spike | L-AspAc | 68.33 | 1.60 | 2.3% | 3.4% |
| | L-AspAc_C3 | 68.03 | 0.99 | 1.5% | -1.8% |
| Asparagus 3 spike | L-AspAc | 73.31 | 0.41 | 0.6% | -4.1% |
| | L-AspAc_C3 | 73.55 | 0.25 | 0.3% | -4.3% |

When comparing the results of the CQ40 sample, the L-AspAc_C3 method has evidently better accuracy, standard deviation, and coefficient of variation than the original L-AspAc method. The spiked potato and asparagus samples show good precision and accuracy, even though the results are not as good as with L-asparagine methods.

In addition to the accuracy tests, the quality controls are measured in three replicates at the beginning of every measuring day to ensure the methods' reliability. Results from quality control runs can also be used to evaluate methods' accuracy. The quality control results of the L-Asp and L-Asp_C3 methods for the QC40 sample are presented in Figure 20. Results cover for a total of eight days.

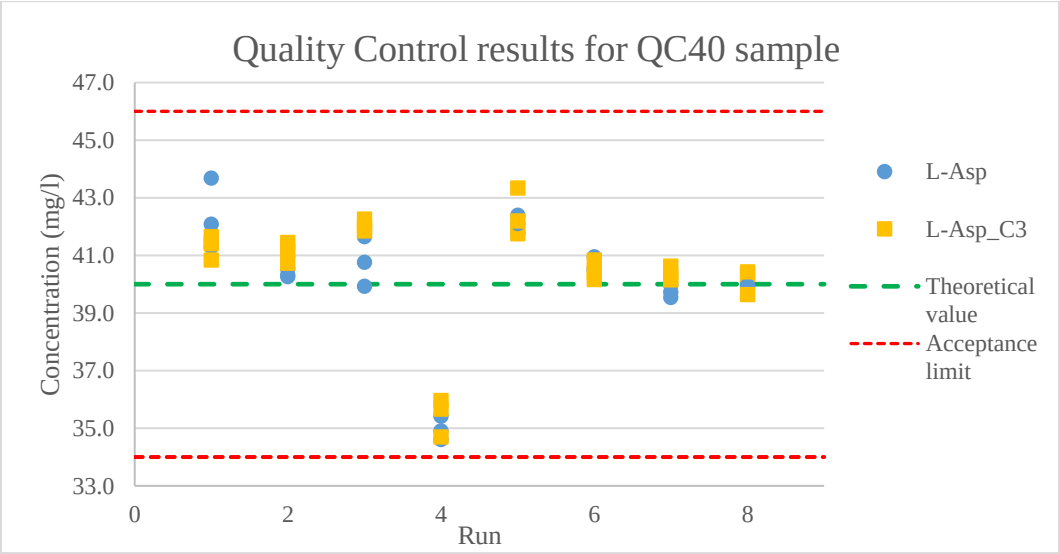


*Figure 20. Quality control results of QC40 samples over eight days.*

The Figure 20 illustrates that the quality control results for QC40 sample are alike for both L-Asp and L-Asp_C3 method. This indicates that the variation between runs can be assumed to be due to external factors, such as, changes in operating condition. Such conditions include, for example, room temperature and humidity. The variation may also be due to an air bubble in a dispenser or human error by the analyst.

## 11.4. Linearity

The linearity measures the reliability of the method across the measuring range. The method is applicable only in such a range where the sample's observed results correlate with its theoretical values. The measuring range is a range in which the correlation between the observed and expected values is linear. As stated previously in Chapter 10.6, the L-Asp method's measuring range consists of the primary and secondary test ranges. The difference between the primary and secondary test range is dilution. While the primary test range has 1+0 dilution, the secondary test range has dilution 1+4. To affirm the linearity of the entire measuring range, it is particularly important to test the method's linearity at the transition point of the test ranges.

Figure 21 illustrates the linearity results obtained for the L-Asp and L-Asp_C1-C3 methods. The results show that all candidate methods are linear over the measuring range and are comparable to the original L-Asp method. The equations presented in Figure 21 illustrate the linearity of the method. The results indicate that the most linear method is L-Asp_C1, and the least linear is L-Asp_C3.

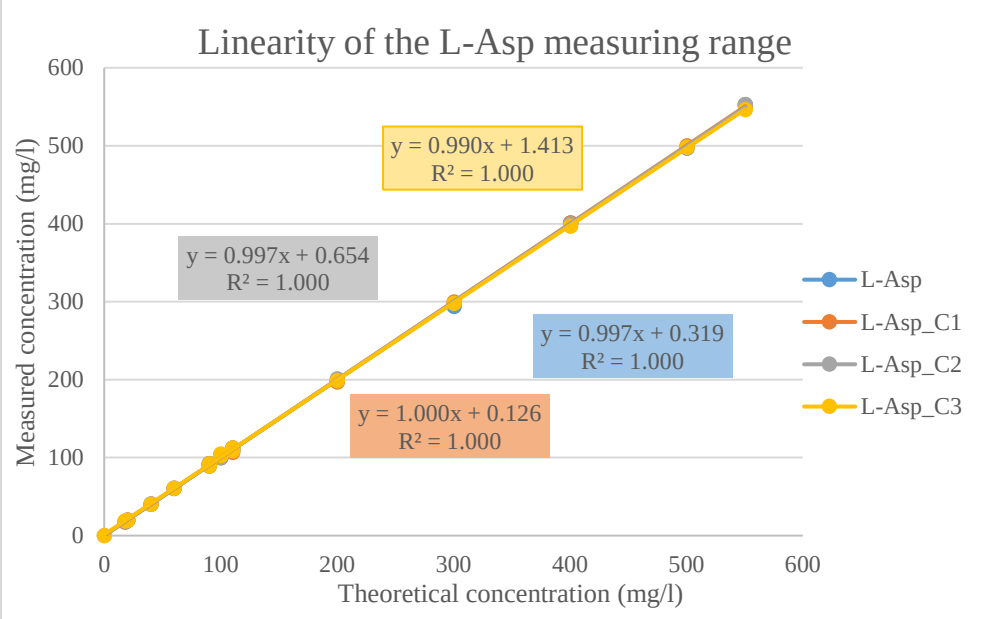


*Figure 21. Linearity of the original L-Asp method and the three candidate methods over the measuring range*

The linearity results for L-Aspartic acid methods are presented in the Figure 22. The results show that the linearity of the candidate method L-AspAc_C3 is better than the linearity of the original L-AspAc method.

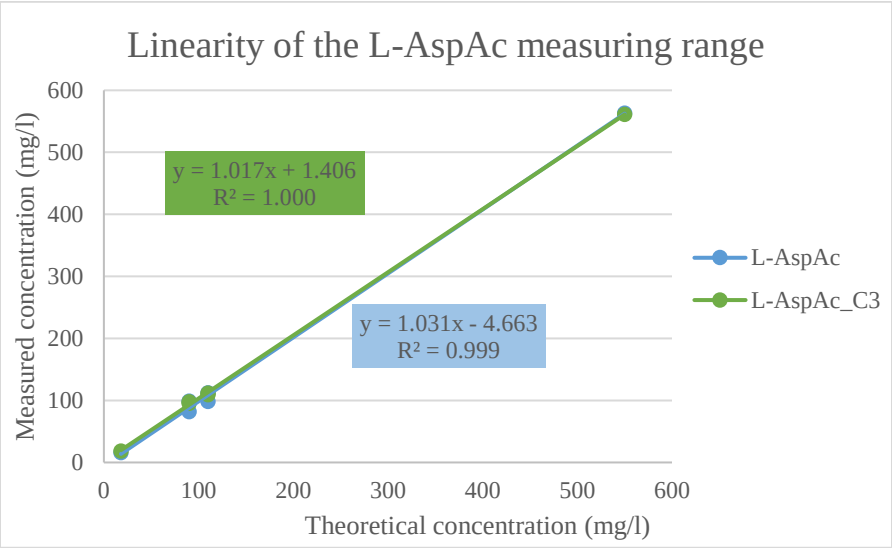


*Figure 22. Linearity of the L-AspAc methods over the measuring range.*

## 11.5. Kinetic measurement

Kinetic measurement measures the response of the sample as a function of time. The measurement aims to examine whether the enzymatic reaction has reached its end before the sample is analysed. The kinetics are measured from the blank and from the reaction according to the applications reviewed in Chapter 10.2. Essentially, the kinetic applications are identical to the original applications, but instead of incubating the sample before the measurement, the kinetic method initiates a kinetic measurement that lasts at least as long as the incubation time. The results of the kinetic measurements are presented in Figure 23.

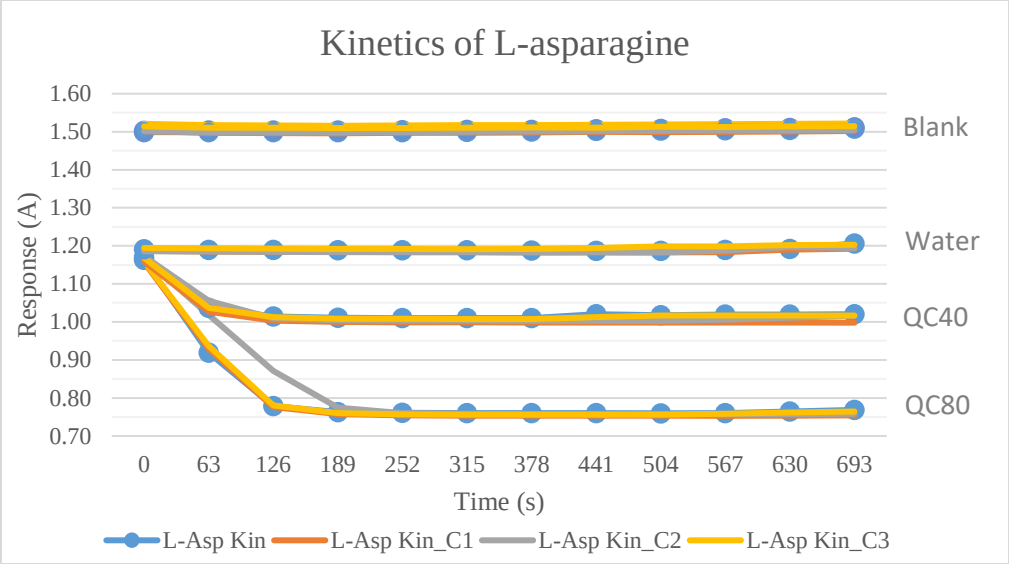


*Figure 23. Results of L-Asp methods reaction kinetic measurements for samples water, QC40 and QC80 alongside with results of blanks kinetic measurements.*

Figure 23 combines the results of reaction kinetics and blank kinetics. The samples used in both kinetic measurements were water, QC40, and QC80. As can be seen from the figure, the sample type does not affect the response when measuring blanks kinetics, as all samples have the same response. On the other hand, apparent differences can be observed between the sample responses in reaction kinetic measurements. The sample response depends on the sample's concentration, and in this case, the lower the response is, the higher the sample concentration is, as the figure illustrates.

All the L-asparagine candidate methods have similar kinetic results to the original L-Asp method. It is also clear that the enzymatic reactions have reached completion before 420 seconds, which is the incubation time used in original applications. Similar observations can be made from Figure 24, which demonstrates the kinetic results of L-aspartic acid methods.

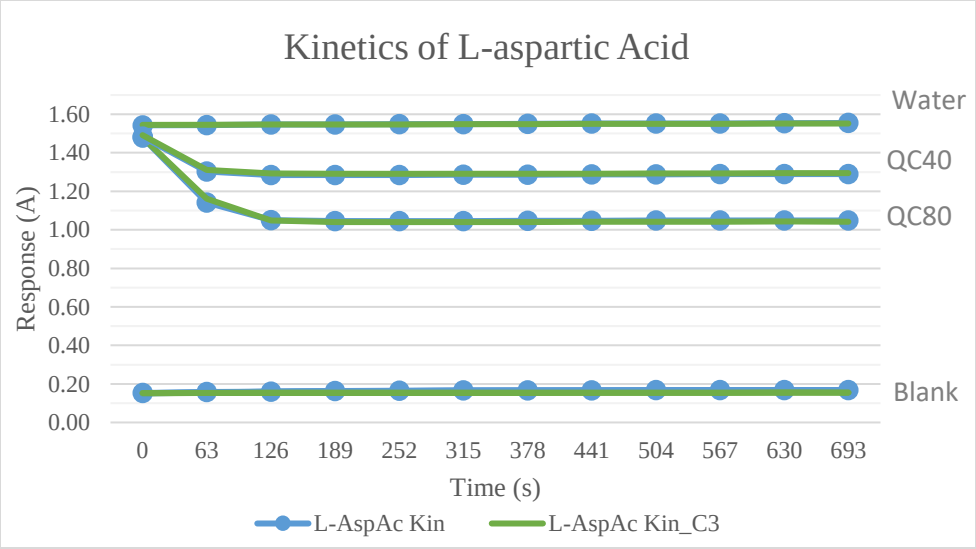


*Figure 24. Results of L-AspAc methods reaction kinetic measurements for samples water, QC40 and QC80 alongside with results of blanks kinetic measurements.*

## 11.6. Spectral comparison

Spectral comparison is used to observe chemical differences between the different methods. Different chemicals have different spectrums, and by observing the differences, the candidate reagent's suitability can be assessed. The spectral comparison was conducted for the original L-Asp R1 reagent and for the three candidate reagents (R1_C1-C3). The measurement was done with a UV-Vis spectrophotometer, which measured the reagent's absorbance as a function of wavelength. The measured spectra are presented in Figure 25, and the inlay graph shows a zoom of wavelength 340, which is the wavelength used to measure L-asparagine and L-aspartic acid.

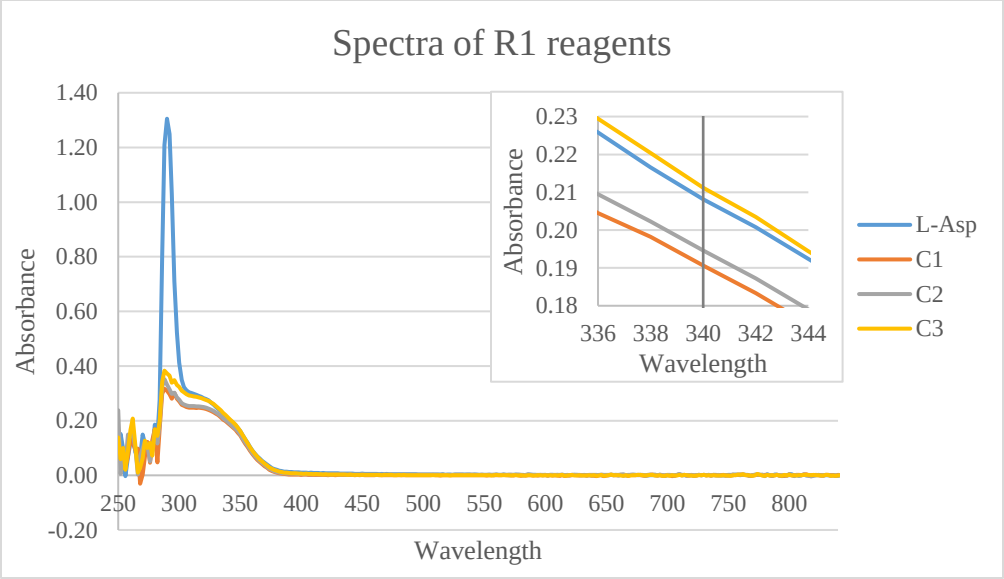


*Figure 25. Spectra of R1 reagents. Inlay graph shows zoom on the interval 336-344 nm.*

The phenyl group of Triton X-100 absorbs strongly at 280-300 nm, as shown by the spectrum of the L-Asp R1 reagent. The modified candidate reagents have otherwise similar spectra as the original reagent, except for the absence of phenyl absorption. The closer examination at 340 nm demonstrates the minor differences between the reagents. Figure 25 shows that of the three candidate methods, the R1_C3 reagent is most similar to the original R1 reagent, while R1_C1 and R1_C2 reagents give a slightly lower absorbance.

The spectral comparison was also done for L-asparagine and L-aspartic acid reactions. The reaction spectra of L-Asp methods were measured from standard solution, which concentration was 100 mg/l. For the L-AspAc methods, the reaction spectra were measured from the QC40 sample. The recorded reaction spectra for L-Asp and L-AspAc methods are demonstrated in Figures 26 and 27, respectively.

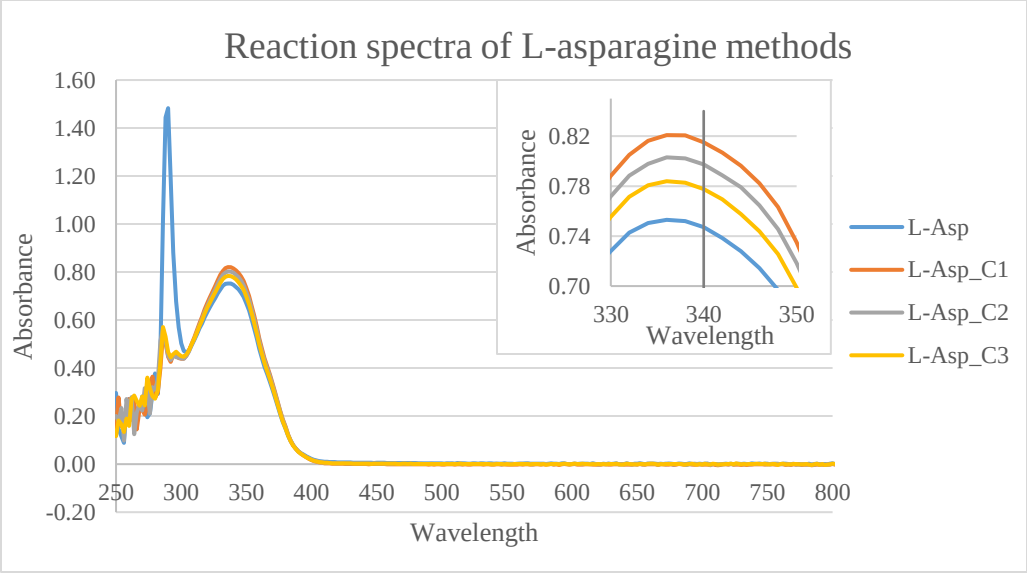


*Figure 26. Reaction spectra of L-asparagine methods measured from std100 sample. Inlay graph shows zoom on the interval 330-350 nm.*

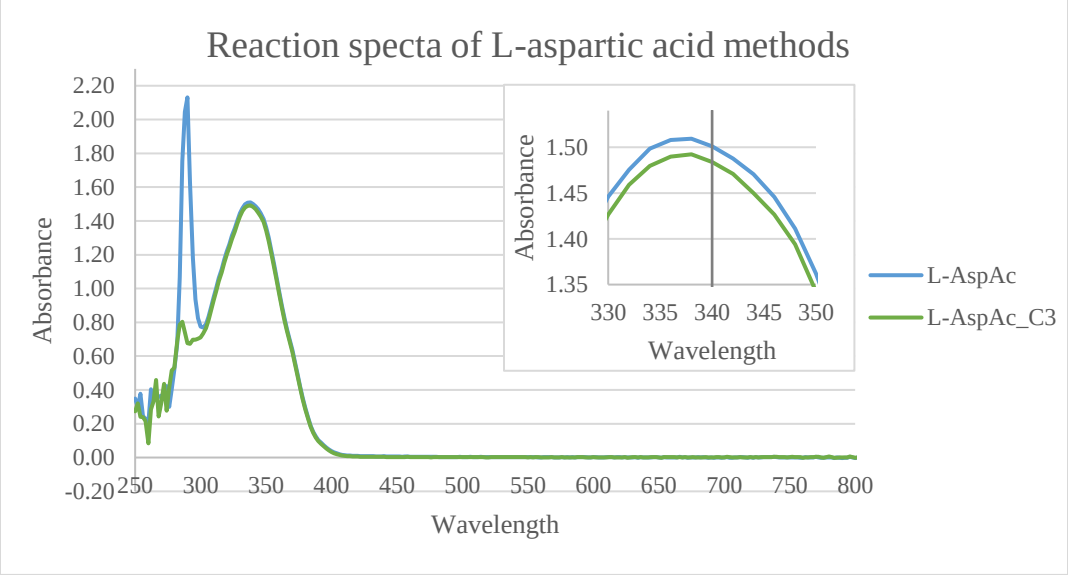


*Figure 27. Reaction spectra of L-aspartic acid methods measured from QC80 sample. Inlay graph shows zoom on the interval 330-350 nm.*

Both Figures 26 and 27 show that the reaction spectra of all candidate methods have a good correlation with the original methods. This indicates that the results obtained with candidate methods will be in line compared to the original method and that modification of the reagents does

not significantly affect the enzymatic reaction. Of all the L-Asp candidate methods, the reaction spectrum of L-Asp_C3 is closest to the original method, as can be seen from the inlay graph in Figure 26. According to these results, all the candidate methods could be chosen to replace the original L-Asp/L-AspAc method.

## 11.7. Selection of the candidate method

As previously discussed, the three candidate methods' performance is very similar to the original method, and all the candidate methods meet all the criteria for feasibility tests. Based on these, any of the three candidate methods could have been selected for replacing the L-Asp/L-AspAc method. However, the method L-Asp_C3 showed several times slightly better performance than the two other candidate methods. This was the case, for example, in calibration curve and spectral comparison. The other reason for selecting the L-Asp_C3 method over other methods was the reagent's easier handling during the preparation of the R1 solution. Lastly, the surfactant chosen for R1_C3 was harmless and didn't have any hazard pictograms or statements. Compared to the other two candidate surfactants that were harmful, corrosive, or hazardous to the environment, the C3 surfactant was chosen with good justifications to be the Triton X-100 replacer.

# 12. Conclusion

The aim of the experimental study was to find an appropriate replacer to Triton X-100 chemical and replace the chemical in an L-asparagine/L-aspartic acid test kit of Thermo Fisher Scientific. The feasibility of three candidate methods were tested to evaluate the candidate method's performance compared to the original L-Asp/L-AspAc method. The three candidates were selected successfully as all the candidates could have been selected to the Triton X-100 replacer according to the feasibility results. The performed feasibility tests included estimation of methods precision, accuracy, and linearity. Also, the method's calibrations, reaction kinetics, and spectra were compared. All the feasibility tests were conducted successfully and with good results.

The surfactant chosen to replace the Triton X-100 in the L-Asp/L-AspAc test kit, was non-hazardous and harmless to health and the environment. This replacement fulfills the ECHAs aim for the authorization process, which intention is to ensure that SVHC substances are replaced by less dangerous substances.

The feasibility results of the chosen candidate L-Asp_C3 were approved and the project was continued by performing validation and verification tests for the L-AspAc_C3 method.

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